MOLECULAR CHARACTERISATION AND PLANT-GROWTH PROMOTION POTENTIAL OF PHOSPHATE SOLUBILISING BACTERIA FROM ROOTS OF SELECTED CROPS AROUND MOROGORO MUNICIPALITY, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN SOIL SCIENCE AND LAND MANAGEMENT OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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EXTENDED ABSTRACT

Soil infertility is reported to be among the most limiting factors for crop production and yield. Despite being abundant in most soils, only a small proportion of phosphorus is readily available to plants due to its high reactivity with soil constituents and slow release from phosphate compounds. Phosphate solubilising bacteria (PSB) play an important role in phosphorus nutrition. These microbes can solubilise various insoluble phosphate compounds through different mechanisms including production of organic and inorganic acids, production of chelating substances and ammonium assimilation, thus enriching soluble phosphorus into soil solution for plant uptake. Use of phosphate solubilising bacteria in agriculture has been reported to increase crop yield in different crops including maize (*Zea mays* L.). Other than phosphate solubilisation, PSB can also solubilise micronutrients including zinc (Zn) and iron (Fe). PSB are also known to produce various plant growth promoting substances such as indole acetic acid (IAA) and siderophore, which are important for crop growth and development. This study therefore aimed at evaluating the plant growth promotion potential of phosphate solubilising microorganisms by, in addition to phosphate solubilisation, looking at their potential for zinc solubilisation, siderophore and IAA production and plant growth promotion in general. Purified colonies of bacteria from 19 native PSB isolated from selected field and garden crops grown around Morogoro municipality, Tanzania, were found to be strong phosphate solubilisers, hence were selected for further studies. Morphologically, these bacteria were whitish, yellowish to creamy in colour, rod shaped and gram negative. Based on 16s rRNA gene sequence most of the isolates were found to belong to the bacterial genus *Burkholderia* while a few others belonged to the genus *Ralstonia*. All isolates were positive for IAA and siderophore production and zinc solubilisation although at varying levels. On phosphate solubilization, *Burkholderia cepacia* strain GPY1 isolated from rice was the most promising strain releasing the highest phosphorus concentration (84.8 mg of soluble P L^{-1}) compared to the lowest

amount (10.85 mg of soluble P L^{-1}) that was released by *Burkholderia territorii* strain KBB5 isolated from rice. Similarly, *Burkholderia cepacia* strain ATCC 25416 isolated from rice was the most promising IAA producer, producing up to 28 mg of L^{-1}, followed by *Burkholderia cepacia* strain GYP1 isolate from sweet potato which released 21 mg L^{-1} of IAA. On the other hand, the lowest IAA amount (i.e. 1.072 mg L^{-1}) was from *Burkholderia territorii* strain S2 isolated from rice. Furthermore, siderophore production as measured in percentage siderophore unit (PSU) was highest (95 %) by *Burkholderia sp.* QN m1 isolated from sweet pepper, followed by *Burkholderia territorii* strain KBB5 (94.82%) and *Burkholderia territorii* strain S2 (93.98%) both isolated from rice, while the lowest percentage siderophore unit was 28.77 % produced by *Burkholderia cepacia* strain GYP1 isolated from sweet potato. The highest quantinty of zinc soulubilised was 347.5 mg of soluble Zn L^{-1} by *Burkholderia territorii* strain KBB5 isolated from sweet potato followed by *Burkholderia cepacia* strain ATCC (242.1 mg L^{-1}) isolated from sweet potato.

Direct application of bacterial cultures to maize seedlings was observed to significantly (P = 0.05) increase both plant height and shoot elongation as compared to a water treated control. Bacteria strains indicated varying abilities in promoting root and shoot elongation. However, strains belonging to *B. cepacia* were the most promising plant growth promoters as compared to other strains. Overall, the finings of this study imply that bacterial isolates can be used as inoculants for enhancing plant growth and consequently yield. However field trials need to be carried out to evaluate the performance of the strains under field conditions.

DECLARATION

I, STEPHEN, GERISON SADDICK, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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DEDICATION

This dissertation is highly dedicated to the Almighty God who gave me the strength, courage, patience, and grace to do and manage this study. To my late father, Saddick Stephen Ntabaje (1965-2017), who fought bravery to make sure that I reach this stage, may your soul rest in peace. You are always missed and remembered.

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AAS	Atomic Absorption Spectrophotometer
Al	Aluminium
Al-P	Aluminium Phosphate
ANOVA	Analysis Of Variance
BLAST	Basic Local Alignment Search Tool
Ca	Calcium
Ca^{2+}	Calcium ion
CAS	Chrome Azurol S
Cm	Centimetre
Co	Cobalt
CO_2	Carbon dioxide gas
CRD	Completely Randomised Design
Da	Dalton
DNA	Deoxyribonucleic acid
et al.	And others
Fe	Iron
Fe-P	Ion Phosphate
g	Gram
h	Hour
H^+	Hydrogen ion
i.e.	That is
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
IITA	International Institute of Tropical Agriculture

L	Litre
Mg	Magnesium
mg	Milligram
Mg^{2+}	Magnesium ion
Mn	Manganese
Mo	Molybdenum
NB	Nutrient broth
NCBI	National Centre for Biotechnology Information
Ni	Nickel
O_2	Oxygen gas
P	Phosphorus
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
pH	Potential hydrogen
PhD	Doctor of Philosophy
PSB	Phosphate Solubilising Bacteria
PSF	Phosphate Solubilising Fungi
PSI	Phosphate Solubilisation Index
PSM	Phosphate Solubilising Microorganisms
PVK	Pikovaskaya
r.p.m.	revolution per minute
Redox	Reduction-Oxidation reaction
rRNA	Ribosomal RiboNnucleicAcid
SUA	Sokoine University of Agriculture
Trp	Tryptophan
Zn	Zinc
ZSI	zinc solubilisation index

μ micro (i.e. $x10^{-6}$)

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Most tropical soils have abundant P. However, most of the P is found as insoluble phosphates of organic and inorganic compounds from which the P is not readily available for plants (Cordell *et al*., 2009; Hirsch *et al*., 2006; Rengel and Marschner, 2005). This makes phosphorus to be the second plant – growth limiting nutrient after nitrogen (Szilas, 2002). In Tanzania most agricultural soils are considered as infertile (Nandwa, 2001). In their study, Szilas *et al*. (2005) reported that major agricultural areas in different ecological zones in the sub-humid and humid areas of Tanzania have soils which are severely weathered and have limited but variable capacities to hold and release nutrients in plant-available forms. Thus, soil infertility seems to be a major cause of reduced crop productivity in the country as reported by Nickson (2017).

Use of inorganic fertilisers has been adopted as a means of combating soil infertility (Amuri *et al*., 2013). However, to overcome P deficiency in agricultural soils, frequent and regular application of phosphate fertilisers are needed (Reddy *et al*., 2002). Tanzania agriculture is dominated by small scale farmers who live and operate under severe financial constraints. Investment in fertilizer use by small scale farmers competes with other uses of their limited financial resources for meeting immediate needs (Senkoro *et al*., 2017). For this reason, alternative and cost-effective approaches to enhance soil fertility must be developed and implemented to ensure food security among small scale and marginal farmers. Use of beneficial microorganisms has been proposed as being a viable biological alternative for sustainable production of crops (Sharma *et al*., 2013).

1.2 Phosphorus and its Availability to Plants

Phosphorus in soils exists in various compounds of organic (Po) and inorganic (Pi) origin which have different behaviour and fate (Hansen *et al*., 2004; Turner *et al*., 2007). Inorganic phosphorus accounts for 35% to 70% of total soil P and covers primary minerals such as apatites, strengite, and variscite (Shen *et al*., 2011), and secondary minerals in form of phosphate salts such as $Ca_3(PO_4)_2$, $FePO_4$ and $ALPO_4$ (Oelkers and Valsami-Jones, 2008). Organic P on the other hand occupies 30% to 65% of the total soil P (Harrison, 1987) and includes stable compounds like inositol phosphates and phosphonates, and active compounds like orthophosphate diesters, labile orthophosphate monoesters, and organic polyphosphates (Condron *et al*., 2005).

The term available-P refers to the amount of soil P that can be extracted from solution and surfaces or taken up by plant roots and utilized by the plant to grow and develop during their life cycle (Setiawati and Handayan, 2010). Most of African tropical soils have inherently small amounts of plant available phosphorus (Oberson and Joner, 2005). Phosphorus is made available to plants through various processes such as weathering of rocks and minerals, fertiliser and manure application and mineralisation of organic P (Turner *et al*., 2007). The effectiveness of these processes varies depending on soil physico-chemical conditions (Shen *et al*., 2011; Hinsinger, 2001). Naturally, the release of soluble phosphorus from its insoluble compounds takes time and is a slow process under natural conditions, which accounts for the low concentration of plant available P (Oelkers and Valsami-Jones, 2008; Pierzynski *et al*., 2005; Shen *et al*., 2011). According to Syers et al. (2008) P concentration in soil solution ranges from very high (i.e. 10^{-4} M), to deficient (i.e. 10^{-6} M), to very low-fertility tropical soils (i.e. as low as 10^{-8} M). In addition to slow-release of phosphorus from its origin compounds, availability of P to plants is also constrained by high reactivity of Pi in soil (Oberson and Joner, 2005; Marschner and Rengel, 2012). Soil pH, which

determines the rate of P availability is also reported to deplete plant available P (Setiawati and Handayanto, 2010) (Figure 1.1). In most cases P is available at nearly neutral pH, 6 to 7.5. At pH greater than 7 P is fixed by calcium and magnesium while at pH less than 6 P is fixed mainly by Fe and Al (Marschner and Rengel, 2012). Intensive cultivation with little or no fertilizer use and poor nutrient management practices also result into shortage of phosphorus (Kimani *et al*., 2003).

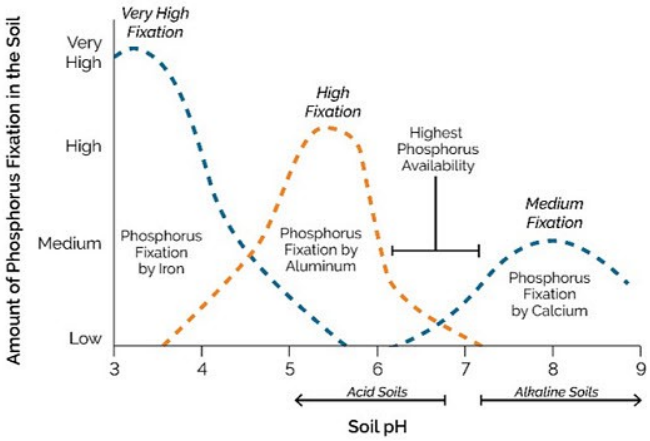

Figure 1.1: The effect of soil pH on phosphorus availability

(Source: Price, 2006).

1.3 The Role of Soil Microorganisms in Enhancing P Availability

Phosphate solubilising microorganisms (PSMs) are group microorganisms capable of solubilizing various inorganic and organic insoluble phosphate compounds thereby contributing to increasing plant available P (Chauhan *et al*., 2017; Chen *et al*., 2006; Manzoor *et al*., 2017). Several fungi and bacteria species have been reported to solubilise insoluble phosphate compounds. Examples of phosphate solubilising bacteria include species of *Burkholderia* (Zhao *et al*., 2014), *Pseudomonas*, *Bacillus*, *Agrobacterium, Micrococcus* and *Flavobacterium*. Fungi include *Aspergillus*, *Penicillium*, *Fusarium*, and *Sclerotium* (Alam *et al*., 2002). There are also reports that actinomycetes belonging to the genera *Actinomyces, Micromonospora,* and *Streptomyces* and algae such as cyanobacteria exhibit P solubilisation potential (Sharma *et al*., 2013). Among the entire microbial population in

soil, P solubilizing bacteria comprise 1–50 % and P solubilizing fungi 0.1 to 0.5 % of the total respective populations (Anand *et al*., 2016).

Nevertheless, protozoa have also been reported to enrich plant available P, both directly and indirectly. Protozoa can directly free phosphorus bound to organic compounds due to their high affinity to vast organic sources including high molecular weight compounds. It has also been reported that P can be released when microorganisms are grazed by microbivores including protozoa (Alphei *et al*., 1996; Cole *et al*., 1978). Other potential microbivores include nematodes (Cole *et al*., 1978). Although microbivores may reduce the effectiveness of P solubilization, they may also stimulate the release of P immobilized in the PSM (Chapuis-Lardy *et al*., 2011).

Soil macrofauna are also known for their potential ability to improve soil available P. Soil macrofauna produce biogenic structures, mainly casts for earthworms and mounds for termites, in which P contents and forms differ from those of the surrounding soil (Chapuis-Lardy *et al*., 2011; Oberson *et al*., 2011). Their overall activity markedly changes P availability in soils where they are active while biogenic structures also impact P transfer by infiltration or runoff when eroded (Chapuis-Lardy *et al*., 2011).

Microbial phosphate solubilisation is a very crucial process in agriculture, as it ensures availability of soluble phosphorus to plants and enables use of a wide range of P sources including insoluble phosphate rocks (Gyaneshwar *et al*., 2002). Other than phosphate solubilisation, PSMs enhances plant growth through production of plant growth regulators such as siderophore and IAA (Richardson *et al*., 2009). PSMs also act as a labile source of soil P. Microbial biomass P contributes significantly to the total soil P and is generally equivalent to, or exceeds, that held in plant biomass (Richardson and Simpson, 2011). The estimated amount of microbial biomass P in bulk soil is

around 2% to 10% of total soil P, and in some cases at different stages of soil development this may be as much as 50% (Oberson and Joner, 2005; Achat *et al*., 2010). Incorporation of phosphorus into microorganisms' cells temporarily decreases soil solution orthophosphate (Oehl *et al*., 2001; Ehlers *et al*., 2010). This is an important mechanism in regulating the P supply in soil solution (Seeling and Zasoski, 1993; Ehlers *et al*., 2010), and prevents P losses through surface runoff and leaching (Gyaneshwar *et al*., 2002) and through soil reactions (adsorption or fixation) (Olander and Vitousek, 2004; Khan and Joergesen, 2009). Studies by Oehl *et al*. (2004) and Bünemann *et al*. (2007) reported that orthophosphate released through microbial turnover contribute significantly to increase in amount of soil P sufficient to gwroth of plants. Microorganisms are therefore an integral part of the soil phosphorus (P) cycle and as such play an important role in ensuring availability of P to plants (Figure 1.2).

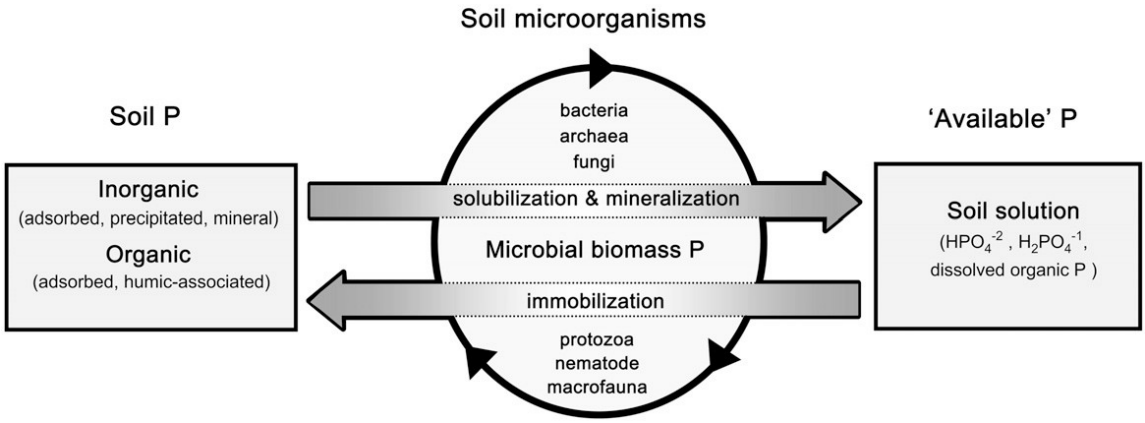

Figure 1.2: Importance of microorganisms to P availability in soil

(Source: Richardson and Simpson, 2011).

1.4 Occurrence and identification of Phosphate Solubilising Bacteria (PSB)

Phosphate solubilising bacteria inhabit diverse ecology; however, they are more concentrated in the rhizosphere. These strains are metabolically more active than when found in other locations (Vazquez *et al*., 2000). Generally, it is urged that one gram of fertile soil contains 10^1 to 10^{10} bacteria (Mohammadi, 2012). Phosphate solubilising bacteria occur in different shapes including cocci (spherical, 0.5 µm), bacilli (rod, 0.5–0.3 µm) or spiral (1-100 µm) (Baudoin *et al*. 2002).

Distribution of the phosphate solubilising bacterial (PSB) populations varies between soils depending on soil properties including physical and chemical properties, organic matter, and P content (Kim *et al*., 1998). According to Yahya and Al-Azawi (1998), large populations of phosphate solubilising bacteria are found in agricultural and rangeland soils.

Phosphate solubilising bacteria have been isolated, tested, identified and consequently applied in agriculture to enhance crop production (Manzoor *et al*., 2017). Unlike in the past when PSBs and other microorganisms were only studied based on microscopic observation or culture-dependent methods, recently, molecular ecology techniques based on sequence comparisons of nucleic acids have been applied in identification and classification of these microorganisms (Schütte *et al*., 2008). The method is accurate and rapid (Henry *et al.*, 2004) and thus streamlines the entire process of identifying specific classes of microorganisms that are potential for production of commercial P solubilising inoculants (bio-fertiliser) (Zaidi *et al*., 2009).

1.5 Mechanisms of Inorganic P Solubilisation

The most effective mechanism governing inorganic phosphate solubilisation is the production of organic acids (Gyaneshwar *et al*., 2002). However, other mechanisms including ammonium assimilation, production of inorganic acids and production of organic chelating substances can also solubilise insoluble inorganic phosphate compounds (Khan *et al*., 2014; Sharma *et al*., 2013). Bacterial-produced organic acids solubilize insoluble phosphates by lowering the pH, chelation of cations and competing with phosphate for adsorption sites in the soil (Khan *et al*., 2010; Nahas, 1996). The carboxylate group from organic acids such as formic, acetic, propionic, lactic, glycolic, fumaric, and succinic acids can chelate the cations such as Ca, Al and Fe bound to phosphate, thereby releasing soluble P (Sharma *et al*., 2013).

The general chemical equation for proton substitution from microbial produced organic acid is presented according to Pradhan and Sukla (2005), as follows:

$(Ca^{2+})\ m\ (PO_4^{3-})\ n + (HA) = (H^+)\ (PO_4)^{3-}\ n + (Ca^{2+})\ m\ (A^-)$ ---------- (i)

According to Staunton and Leprince (1996), carboxtlate containing compounds produced by PSB have high affinity to calcium and solubilize more phosphorus than acidification alone. Acidification of medium causes the release of adsorbed phosphorus especially in basic conditions (Villegas and Fortin, 2002).

Beside the organic acids, the pH of media can be lowered through various microbial mediated activities including production of protons through ammonium assimilation (Schaechter, 2009), and gaseous (O_2/CO_2) exchanges (Mohammadi, 2012). Inorganic acids such as hydrochloric acid can also solubilize phosphate by lowering the pH and chelating activities of associated hydroxyl groups, but they are less effective as compared to organic acids (Sharma *et al*., 2013). Concentrating protons through acidification causes the substitution of phosphate-bound cations such as Ca^{2+} and Mg^{2+} (from phosphate adsorption site) by H^+ thereby releasing soluble phosphate (Villegas and Fortin. 2002). This mechanism is reported to occur especially in alkaline soils where soil phosphates, mainly the apatites and metabolites of fertilizers, are fixed in the form of calcium phosphates (Mohammadi, 2012). In acidic soils solubilisation of $FePO_4$ and $AlPO_4$ occurs mainly by carboxylic acids (Henri *et al*., 2008; Khan *et al*., 2007) through direct dissolution of mineral phosphate as a result of anion exchange of PO_4^{3-} by acidic anion, or by chelation of both Fe and Al ions associated with phosphate (Omar, 1998). Furthermore, carboxylate replace phosphate from sorption complexes by ligand exchange (Whitelaw, 2000) and chelate both Fe and Al ions

associated with phosphate, thereby releasing soluble phosphorus. Reactions between insoluble Ca_3PO_4 with inorganic acids can be ewritten as follows:

$Ca_3PO_4 + 2H_2SO \rightarrow CaH_4(PO_4)_2 + 2CaSO_4$ ---------- (ii)

$Ca_3(PO_4) + 4HNO_3 \rightarrow CaH_4(PO_4)_2 + 2Ca(NO_3)_2$ ---------- (iii)

1.6 Zinc Solubilisation

Zinc is one of the essential micronutrients required in relatively small quantinties (5-100 mg kg^{-1}) in tissues for healthy growth and reproduction of crops. It takes part in enzyme systems as co-factor and as metal activator of many enzymes (Babu *et al*., 2017; Mumtaz *et al.*, 2017). Zinc deficiency in plants retards several physiological processes including photosynthesis, carbohydrate and phytohormone synthesis and nitrogen fixation. Deficiency also causes a reduction in flowering and fruit development as well as affecting crop maturity, leading to decrease in crop yield and nutritional quality of grain (Mumtaz *et al.*, 2017). Some phosphate solubilizing bacteria have been reported to solubilise insoluble zinc compounds including zinc carbonate, zinc oxide and zinc phosphate (Babu *et* al., 2017; Mumtaz *et al*., 2017, Zaheer *et al*., 2019). Production of organic acids is the major mechanisms for zinc solubilisation; however, other mechanisms including production of inorganic acids and chelating substances have also been reported to solubilize insoluble zinc compounds (Fasin *et al*., 2017).

1.7 IndoleAcetic Acid (IAA) Production

Indole acetic acid (IAA) is an important physiologically active auxin. IAA stimulates root cell elongation by modifying certain conditions like increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, increase in cell wall synthesis and inducing specific RXA and protein synthesis (Zhao, 2010). IAA improves water and nutrient uptake

by plants by stimulating primary root growth, lateral root formation, and root hair development (Fukaki and Tasaka, 2009). In some plants IAA inhibits or delay abscission of leaves and induces flowering and fruiting (Zhao, 2010). Microbial IAA biosynthesis is carried out either through tryptophan (Trp)-independent or tryptophan-dependent pathways. In Trp-dependent pathways, tryptophan is solely used as a precursor for IAA production. During the processes microbes use a tryptophan-2-monooxygenase (iaaM) to convert tryptophan to indole-3-acetamide (IAM), which is subsequently hydrolyzed into IAA by the indole-3-acetamide hydrolase (iaaH) (Chandler, 2009). Examples of bacteria exhibiting Trp-dependent process include *Azospirillum brasilense* (Carreno-Lopez *et al.*, 2000). On the other hand, in Trp-independent IAA biosynthesis, indole-3-glycerol phosphate is the likely precursor, and the pathway proceeds in the absence of tryptophan (Normanly *et al*., 1993; Zhao, 2010).

1.8 Siderophore Production

Siderophores (derived from the Greek meaning “iron carriers”) are low molecular weight (200-2000 Da), extracellular organic chelators with a very high and specific affinity for Fe (III) (Ngamau *et al*., 2014). Siderophore not only enhances iron uptake by plants (Braud *et al,* 2009) but it is also known for its biocontrol activities (Ren *et al*., 2005). Siderophore can also chelate and enhance uptake of other micronutrients such as Mo, Mn, Co and Ni. However, due to high cellular demand and low availability of iron, siderophore reacts more with Fe(II) or Fe(III) than with other metals (Bellenger *et al*., 2008; Braud *et al*, 2009). Siderophore is also considered as biocontrol agent due to its suppression of soil-borne plant pathogens. It has been suggested that the siderophore mediated competition for iron with soil-borne pathogens is an important mechanism for biological control as plants are able to use bacterial iron siderophore complexes as a source of iron from soil.

1.9 Potential Use of PSB as Bio-fertilisers in Agriculture

Current soil management practices which involve intensive and sometimes imbalanced use of inorganic fertilisers have been reported to cause several detrimental effects in agriculture, human health and environment (Ju *et al*., 2018). The use of bio-fertilisers has been proposed as the best alternative approach to chemical fertilisers in increasing soil fertility and crop production and yield (Ju *et al*., 2018). Bio-fertilisers are defined as large population of specific microorganisms, or group of beneficial microorganisms, for enhancing the productivity of the soil either by fixing atmospheric nitrogen or by solubilizing soil phosphorus or by stimulating plant growth through the synthesis of growth promoting substances (Rajan, 2002). Bio-fertilizers serve greatly in correcting various constraints associated with inorganic fertilisers since they do not contain traces of hazardous and poisonous materials. Bio-fertiisers are cost effective, eco-friendly and convenient to use safely (Sinha *et al*., 2010). In crop production, bio-fertilisers enhance nutrient availabilities, crop protection against pathogens, improve nutrient and water uptake and stimulate crop growth through production of plant growth promoting substances like hormones, vitamins, and amino acids. Furthermore, bio-fertilisers reduce the huge amount of foreign exchange invested in the importation of synthetic fertilizers, and thereby compensate for the high price of inorganic fertilisers and, thus, enable small-scale farmers to increase their crop yields (Igiehon *et al*., 2017; Mahanty *et al*., 2017; Mugabe *et al*., 1994). Application of high doses of inorganic fertilisers can also be reduced through the concomitant use of bio-fertilisers. A study conducted by Sundara *et al*. (2002) showed a reduction of inorganicl fertilizer (superphosphate and rock phosphate) dose requirement by 25–50% when the fertilisers were used in combination with the indigenous PSB. Due to the fact that about 60% to 90% of the total applied inorgabic fertilizer is converted into plant unavailable form, use of bio-fertilizers can be an important component of intergrated nutrient management systems for sustaining agricultural productivity and a healthy environment (Adesemoye and Kloepper, 2009).

1.10 Problem Statement and Justification

Although some plants are well adapted to low phosphorus availability (Richardson *et al*., 2009) production of most crops remain constrained by P deficiency in soils (Baldotto *et al*., 2012; Oberson

et al., 2006; Szilas, 2002). Inorganic phosphate compounds have been used in agriculture to enhance plant growth and production. These vary from water soluble compounds such as Triple Superphosphate to insoluble rock phosphate (Yingben *et al*., 2012). Low P concentration in most soils calls for judicious use of inorganic fertilizers especially water soluble fertilisers to enhance crop production. However, in Tanzania, this approach is constrained by high price of fertilisers (Reddy *et al*., 2002). Other than high price, inaccessibility of fertilisers, soil fixation of added phosphorus and erratic and unprofitable crop responses to P fertilisers have been reported to reduce the efficiency and use of P fertilisers (Setiawat and Handayanto, 2010; Vassilev and Vassileva, 2003). To overcome such challenges, use of soil microorganisms in crop production has been suggested since they are cost effective and environmental friendly (Khan *et al*., 2009). According to Khan *et al.* (2007), phosphate solubilising bacteria enables plants to access soluble phosphate in a friendlier environment and in a sustainable manner. In Tanzania, there is no specific study conducted to evaluate plant growth promoting abilities by locally isolated phosphate solubilising bacteria. Some few studies, however, have reported on potential abilities of soil microorganisms (fungi and bacteria) to solubilise insoluble phosphate compounds (Simfukwe and Tindwa, 2018), but little is known about plant growth promotion potential of locally isolated phosphate solubilising bacteria. Therefore, this study was undertaken to evaluate phosphate solubilising abilities and plant growth promotion potential of bacteria isolated from root surfaces of selected field and garden crops around Morogoro municipality, Tanzania.

1.11 Objectives

1.11.1 Main objective

The main objective of the study was to determine the potential of microorganisms from soils of different location in Morogoro, Tanzania, in solubilising plant nutrients and improving maize growth.

1.11.2 Specific objectives

i. To isolate and undertake molecular characterisation of phosphate solubilising bacteria from root surfaces of selected field and garden crops around Morogoro municipality, Tanzania.

ii. To determine the ability of the P solubilising microorganisms to solubilise insoluble zinc compound.

iii. To determine the production of plant-growth promoting substances by these microorganisms

1.12 Organisation of the Dissertation

Chapter one: This chapter covers the general introduction providing theoretical background information of the study, literature review with respect to phosphate solubilising bacteria, plant growth promoting potential, justification and objective of the study.

Chapter two: This chapter covers isolation and molecular characterisation of phosphate solubilising bacteria from root surfaces of selected field and garden crops around Morogoro municipality, Tanzania. The chapter covers morphological characterisation and molecular identification of bacteria to species level. A draft paper for this chapter by Stephen, G. S., Tindwa, H. J. and Semu, E. tittled Isolation and molecular characterisation of phosphate solubilising bacteria from root surfaces of selected field and garden crops around Morogoro municipality, Tanzania has been prepared and submitted to the *Journal Tropical of Ecology*.

Chapter three: This chapter covers evaluation of plant growth promoting potential of bacteria isolated from root surfaces of selected field and garden crops around Morogoro municipality, Tanzania. This chapter covers assays of plant growth promoting traits, indole acetic acid (IAA) production, siderophore production, zinc solubilisation and phosphate solubilisation. It also covers evaluation of the effect of bacterial inoculation on the growth of maize (*Zea mayse*) as a test crop. A draft paper for this chapter by Stephen, G. S., Tindwa, H. J. and Semu, E. tittled Plant growth

promoting potential of bacteria isolated from root surfaces of selected field and garden crops around Morogoro municipality, Tanzania, is under preparation

Chapter four: Is the general conclusions and recommendations. In this chapter key issues are concluded in relation to bacterial potential to enhance plant growth. Also, basic recommendations on the study are being highlighted.

1.13 References

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CHAPTER TWO

2.0 ISOLATION AND MOLECULAR CHARACTERISATION OF PHOSPHATE SOLUBILISING BACTERIA FROM ROOT SURFACES OF SELECTED FIELD AND GARDEN CROPS AROUND MOROGORO MUNICIPALITY, TANZANIA.

Abstract

Phosphate solubilising bacteria (PSB) serve an important role in the P nutrition of crops. The objectives of this study were to isolate and characterise phosphate solubilising bacteria from root surfaces of different field and garden crops grown around Morogoro municipality, Tanzania. A total of 42 isolates indicated phosphate solubilising activities on a solid Pikovskaya (PVK) medium. Only 19 isolates with strong halo zones were selected for further studies including qualitative and quantitative phosphate solubilisation and molecular identification to species level. Quantitative phosphate solubilisation assay was carried out on the third and ninth days of incubation in order to determine phosphate solubilising ability of bacterial strains with increased period of incubation. Incubation experiments were laid out in triplicates in the Completely Randomized Design (CRD). Raw data were subjected to analysis of variance (ANOVA) using the GenStat Discovery 15th edition software, and treatment means were ranked using the Duncan's Multiple Range Test at 5% probability (P = 0.05). Morphological characterisation showed that most of the bacterial isolates were rod shaped and gram negative. Molecular characterisation based on 16s rRNA gene sequence techniques revealed that bacteria belonged to the genera *Burkholderia* and *Ralstonia*. Qualitative results indicated that the Phosphate Solubilisation Index (PSI) significantly varied (P = 0.05) betweem isolates, and ranged from a minimum of 4.145 to a maximum of 7.083. Also, quantitative analysis showed significant variations (at P = 0.05) in phosphate solubilisation among isolates. Furthermore, it was observed that phosphate solubilisation varied with advanced incubation period. Generally, the maximum soluble P was 84.8 mg L^{-1}, registered by *Burkholderia cepacia* strain

GPY1, on the third day of incubation, while *Burkholderia territorii* strain KBB5 indicated the lowest P solubilisation, 10.85 mg L^{-1}, on their third day of incubation. The potential ability to solubilise insoluble tri-calcium phosphate reported in this study gives a clue for future use of these strains in improving uptake of phosphorus by plants in crop production. However, field trials must be perfomed to augment the current findings.

Key words: Phosphate solubilising bacteria (PSB), phosphorus, Pikovskaya's medium (PVK) *Burkholderia*, *Ralstonia*.

2.1 Introduction

Phosphorus is the second major nutrient, after nitrogen, required in large quantities by plants for growth and development (Bai *et al*., 2014; Shahid *et al*., 2015). P is incorporated into macromolecules which are involved in various metabolic processes including photosynthesis, energy transfer, signal transduction and respiration (Bevers *et al*., 2016; Khan *et al*., 2010) as well as nitrogen fixation in legumes (Kouas *et al*., 2005). Despite the fact that most agricultural soils have abundant phosphorus reserves, phosphorus availability to plants remains to be one of the most limiting factors for plant growth mainly because most phosphate compounds exist in soils as insoluble complexes and/or precipitates that have low phosphorus availability for plant uptake (Gyaneshwar *et al*., 2002; Sharma *et al*., 2013; Zhang *et al*., 2017). In Tanzania, over 50 % of cultivated soils are estimated to be P-deficient (Ndungu-Magiroi *et al*. 2014). Inorganic phosphate fertilisers have been used as a major means of replenishing phosphorus to soils (Amuri *et al*., 2013). However, these fertilisers must be applied regularly in order to compromise phosphorus requirement by plants with P sorption on soil surface and precipitation by free $Al^{3+}$and Fe^{3+} in the soil solution (Nziguheba *et al*., 2016). High cost of inorganic fertilisers is a major constraint for small scale farmers in meeting judicious fertilisation (Senkoro *et al*., 2017). Furthermore, high reactivity of phosphorus with soil constituents limits efficiencies of inorganic fertilisers even when water soluble fertilisers like Triple Superphosphate are used (Setiawat and Handayanto, 2010; Vassilev and Vassileva, 2003).

Phosphate solubilization undertaken by phosphate solubilising microorganisms (PSMs) has been proposed as an alternative approach aimed at enhancing the availability of the otherwise insoluble phosphate resources to plants (Wang *et al*., 2017). Inorganic phosphate solubilisation by PSMs involves different mechanisms including production of organic and inorganic acids, release of chelating substances which chelate cations to form stable complexes and thereby freed phosphate

which becomes available for plants, ammonium assimilation and redox activities (Khan *et al*., 2009; Mohan Singh *et al*., 2011; Sharma *et al*., 2013). Different bacteria capable of solubilising insoluble phosphate compounds have been reported by some researchers (Mohan Singh *et al*., 2011; Wang *et al*., 2017; Li *et al*., 2017; Zhang *et al*., 2017; Simfukwe and Tindwa, 2018). The present study aimed at isolating and molecular identification of phosphate-solubilising bacteria from root surfaces of selected crops around Morogoro municipality, Tanzania.

2.2 Materials and Methods

2.2.1 Sample collection and isolation of phosphate solubilising bacteria

Root systems of Irish potato, sweet pepper and rice plants were collected from different areas within Morogoro municipality, Tanzania. Samples were collected from SUA model training farm- main campus, Mazimbu, Kasanga and Kihonda areas within the municipality (Table 2.1). Two plant root samples were randomly collected by carefully uprooting the target plant to recover the entire root system as intact as possible. Roots samples collected were placed into plastic bags, labelled and transported to the microbiology laboratory of the Department of Soil and Geological Sciences of the Sokoine University of Agriculture for further studies.

Prior to isolation, root surface soils were aseptically scraped off each root system using a thin sterile razor blade. Thin layers of root surfaces (epidermis from each sample) were aseptically peeled out and placed onto separate sterile aluminium foils. One g of peeled root surface samples was aseptically transferred into a sterile conical flask containing 100 mL of sterile distilled water to make the 10^2 dillution. Samples were homogenised by shaking for 30 minutes using a shaking incubator at 120 rpm. one mL from serial dilution 10^2 was aseptically transferred into bottles, each containing 9 mL sterilised distilled water to make the 10^3 dilution. Serial dilutions were repeated to make 10^4, 10^5 and 10^6 dilutions. 100 µL aliquots from each of the serial dilutions 10^3, 10^4 and 10^5

were subjected to the spread plate technique (Ponmurugan and Gopi, 2006) on Pikovskaya's agar medium containing 0.5 g yeast extract, 10 g glucose, 5 g $Ca_3(PO_4)_2$, 18 g agar, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.0001 g $FeSO_4.7H_2O$ and 0.0001 g $MnSO_4.H_2O$ in 1 L of distilled water (Pikovskaya, 1948). Inoculated plates were incubated up-side down, at 28 °C, for 5 days for bacterial colonies to grow. Bacterial isolates showing a clear zone around a growing colony were the phosphate solubilising bacteria (Bharucha *et al*., 2013). These were further purified by repeated sub-culturing onto the PVK agar medium.

Similarly, bacteria contained in a commercially available bio-fertilizer product (RizoFos maize manufactured by Rizobacter S.A. SENASA, Argenntina) were isolated following the same procedure as above using the same PVK solid medium.

2.2.2 Qualitative and quantitative determination of Phosphate solubilisation

Bacterial isolates were screened for their abilities to solubilize insoluble phosphate sources (tri-calcium phosphate) in both agar and broth PVK medium. Experiments were laid out in triplicates in the Completely Randomized Design (CRD). Qualitative phosphate solubilisation assay involved spot inoculation of a purified colony of each bacterium at the centre of the PVK agar plates and incubated upside-down at 28 °C. Phosphate solubilising index (PSI) was calculated according to Liu *et al*. (2015) as

$$(PSI) = \frac{Colony\ diameter + Halo\ zone\ diameter}{Colony\ diameter}$$

For quantitative phosphate solubilisation, measurements were done as follows: a loop full of each pure culture was aseptically inoculated into separate 90 mL of previously sterilized PVK broth medium, followed by incubation in a shaking incubator at 150 rpm and 28 °C for 9 days. Non-inoculated medium control contained same amount of PVK broth (90 mL) was incubated similarly. The amount of phosphorus released was quantified at the third and ninth days using the colorimetric

method as described by Okalebo *et al.* (2002). Briefly, the bacterial culture was harvested by centrifugation at 10 000 rpm for 10 minutes. 0.1 mL of the supernatant was added into a 50 mL volumetric followed by the addition of 10 mL of distilled water and 4 mL of colour reagent (phosphate reagent) and the volume was made to 50 mL by adding distilled water. Phopshate reagent was prepared by mixing 500 mL of 2.5 M H_2SO_4, 50 mL of 0.005 M potassium antimonyl tartrate trihydrate, 150 mL of 0.04 M ammonium molybdate tetrahydrate solution and 300 mL of 0.032 M ascorbic acid solution (Margesin and Schinner, 2005). The mixture was left for 20 minutes for blue colour to develop thereafter the intensity of the colour was quantified at 880 nm wavelength using a UV-VIS spectrophotometer. Absorption of analyte at 880 nm wavelength was fitted on the standard calibration curve prepared from KH_2PO_4 standards.

2.2.3 Morphological characterisation and molecular identification of bacterial isolates

Morphological characterisation involved both macroscopic and microscopic observation. Macroscopic features were studied using necked eyes while microscopic studies involved preparation of bacterial smears based on standard Gram stain procedure (Carter and Cole, 2012). Molecular identification of isolates was preceded by a PCR reaction which was performed with initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C and extension for 45 s at 72 °C. Final extension was held for 5 min at 72 °C. Purified PCR products were then sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa for sequencing based on the bacterial 16s rRNA gene sequencing technique (Li *et al*., 2017). All products were sequenced using universal forward and reverse primers namely 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), respectively. The resultant nucleotide sequences were compared with other sequences at the GenBank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST using BLASTn tool to establish the identity of the isolates. A phylogenetic tree was constructed using CLUSTAL X program (Thompson *et al*., 1997), which involved sequence alignment by the neighbour joining method (Saitou and Nei, 1987) and maximum parsimony using the MEGA5 program (Kumar *et al*., 2001).

Grouping of sequences was based on confidence values obtained by boot strap analysis of 1000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using the Kimura two parameter model (Kimura, 1980). Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees (Walpola and Yoon, 2013).

2.4 Results

2.4.1 Isolation and screening of phosphate solubilising bacteria

Isolates were categorised as phosphate solubilising microbes or otherwise based on their ability to form a halo zone on a solid PVK medium (Plate 2.1). Only those with appreciably big halo zones around their respective colonies (Table 2.1) were picked for further characterization.

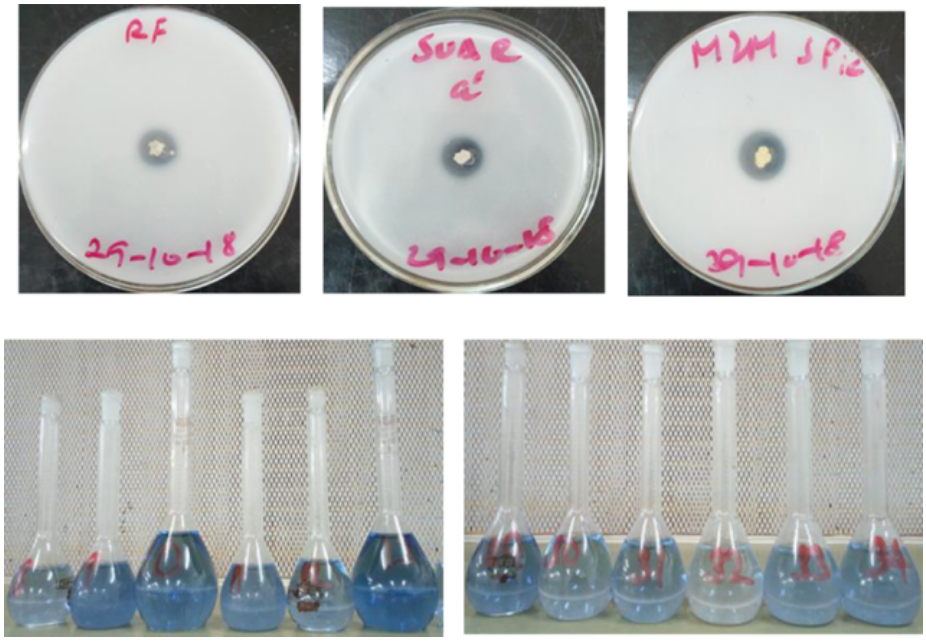

Plate 2.1: Top: presence of clear zone at 7 days after incubation as an indicator of phosphate solubilisation on solid PVK medium. Pure colony of each bacteria was spot inoculated at the center of PVK agar medium and incubated at 28 ºC were for 7 days. **Bottom: the Formed blue colour after 24 h of incubation** as an indicator of phosphate solubilisation in liquid PVK medium.

Table 2.1: Isolate selected for further characterisation

Isolate	Source of bacteria
MZM SPo	Sweet pepper root system from Mazimbu
MZM SPr	Sweet pepper root system from Mazimbu
MZM SPk	Sweet pepper root system from Mazimbu
MZM SPh	Sweet pepper root system from Mazimbu
MZM R12	Rice root system from Mazimbu
KH Rc	Rice root system from Kihonda

KH Rj	Rice root system from Kihonda
KH Ra	Rice root system from Kihonda
MZM R211	Rice root system from Mazimbu
KS PTn	Irish potato root system from Kasanga
KS PT2	Irish potato root system from Kasanga
KS PT4	Irish potato root system from Kasanga
KS PTx	Irish potato root system from Kasanga
KS PT5	Irish potato root system from Kasanga
KS PTk	Irish potato root system from Kasanga
KS PTg	weet potato root system from Kasanga
SUA R3	Rice root system from SUA farm
18 SUA R1	Rice root system from SUA farm
1 SUA R111	Rice root system from SUA farm
RF	Bio-fertiliser

Results on qualitative phosphate solubilisation as presented in Fig. 2.1 showed that the maximum phosphate solubilising index (PSI) was attained by bacterial isolate MZM SPh later identified as *Burkholderia sp.* ON_m1 (section 2.4.2) whereas bacterial isolate KH Rc indicated the lowest phosphate solubilising index (4.145). Bacterial isolate from commercial bio-fertiliser also indicated high phosphate solubilizing index (5.377), which was significantly higher (P = 0.05) than PSI values registered by some native isolates (Fig. 2.1). The formation of blue colour in aliquot supernatatnt solution (Plate 2.1) indicated phosphate solubilisation in broth medium. Results indicate significant (P = 0.05) difference between species and strains. The maximum concentration of soluble P (84.8 mg of soluble P L^{-1}) was registered on the third day by KH Ra isolated from rice while the lowest concentration was 10.85 mg of soluble P L^{-1}registered on the third day by MZM R211. On the other hand, isolate RF isolated from bio-fertilisers indicated the maximum concentration of 50.1 mg L^{-1}on the ninth day (Figure 2.2).

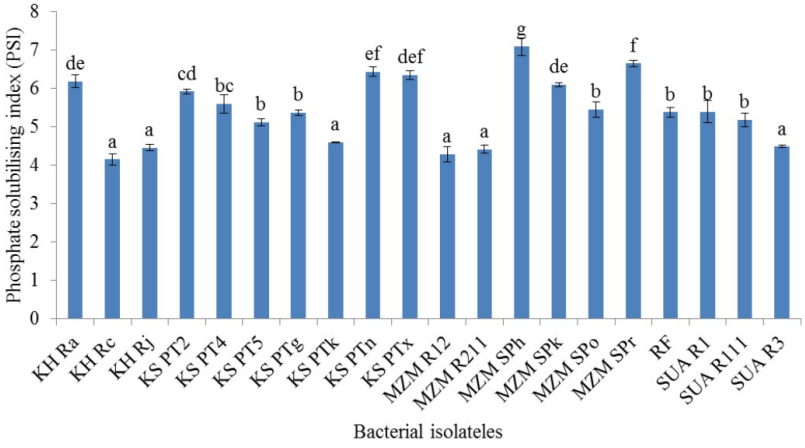

Figure 2.1: Phosphate solubilisation index (PSI) values for bacterial isolates on solid PVK agar medium. Each isolate was inoculated at the centre of a PVK agar plate and incubated for five days. PSI was calculated by taking the total of halozone and colony diameters divided by the colony diameter in centimetres. Bars carrying different letters or combination of letters are significantly (P = 0.05) different from one another according to the Duncan's New Multiple Range Test.

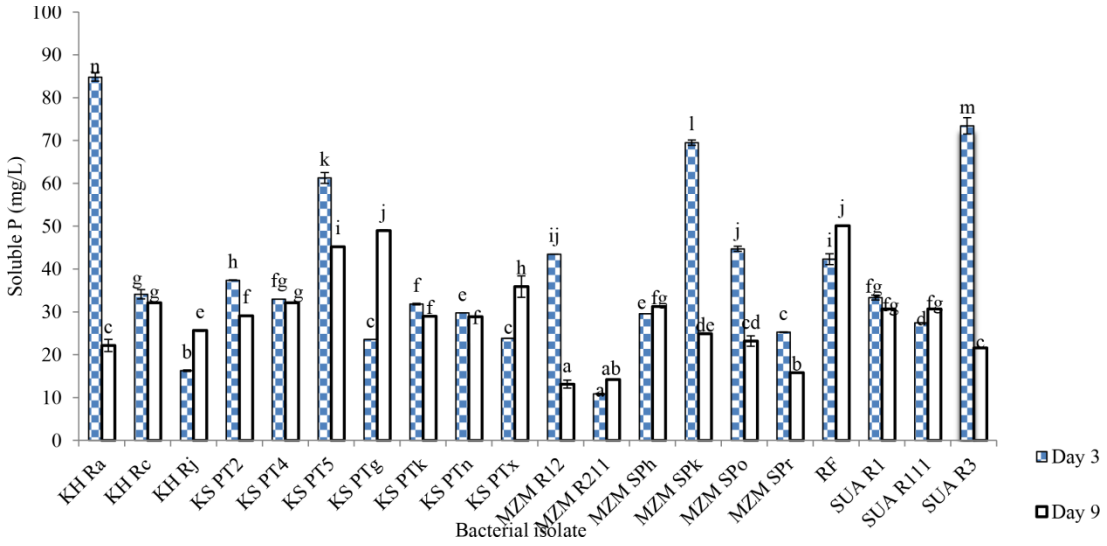

Figure 2.2: Quantitative phosphate solubilisation by various bacterial isolates in PVK liquid medium. Each isolate was inoculated in a PVK broth and incubated in a reciprocating shaking incubator at 150 rpm and 28˚ C for up to 9 days. Amount of soluble P was quantified at 880 nm wave length with UV- VIS spectrophotometer after the development of the blue colour complex following a procedure described by Okalebo *et al.* (2002). Bars carrying different letters or combination of letters are significantly (P = 0.05) different from one another according to the Duncan's New Multiple Range Test.

2.4.2 Morphological and molecular characterisation of phosphate solubilising bacteria

Results of morphological characterization indicated that bacterial colonies were cocci to rod shaped, shiny on the surface, whitish, creamy, to yellow in colour. Microscopic analysis of bacterial smears

indicated that some of the isolates were gram positive while others were gram negative (Table 2.2). Representative micrographs of representative gram positive and negative bacteria are presented in Plate 2.2.

Table 2.2: Morphological characterisation of bacterial isolates

Isolate	Morphological characterisation		
	Colour	**Shape**	**Gram staining**
MZM SPr	Yellow	Rod	Negative
KS PTx	Creamy	Rod	Negative
KS PTg	White	Rod	Negative
KS PTn	Creamy	Rod	Negative
KS PT5	White	Rod	Negative
KS PT4	White	Rod	Negative
SUA R1	White	Rod	Negative
RF	White	Rod	Negative
SUA R3	Creamy	Rod	Negative
KS PT2	Creamy	Rod	Negative
KH Rc	Dark purple	Cocci	Positive
KH Ra	White	Rod	Negative
MZM SPo	White	Rod	Negative
MZM SPk	White	Rod	Negative
MZM SPh	White	Rod	Negative
KH Rj	White	Rod	Negative
MZM R12	White	Rod	Negative
KS PTk	Creamy	Rod	Negative
SIA R11	White	Rod	Negative
MZM R211	White	Rod	Negative

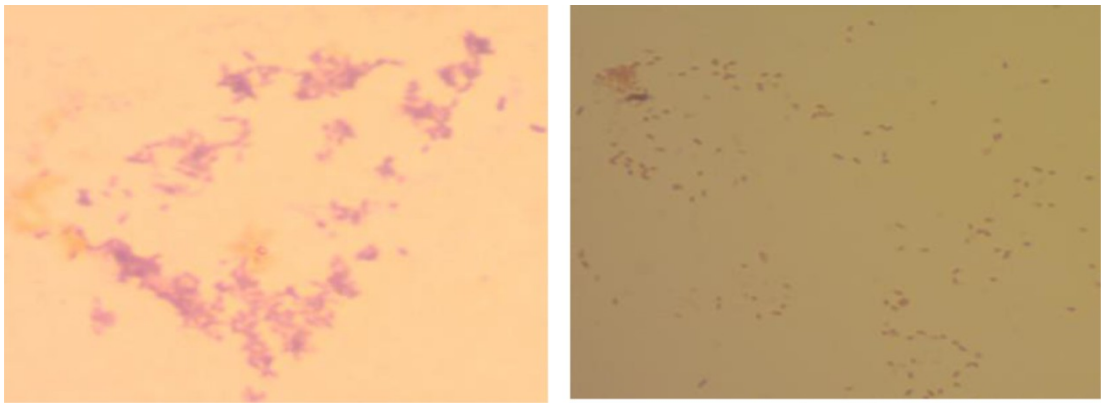

Plate 2.2: Micrographs of representative phosphate-solubilising bacteria. Purple colour indicates gram positive bacteria while pinkish colour indicates gram negative bacteria

Blastn analysis of the 16S rRNA sequences of isolated strains showed high homology (> 90%) of the isolates to available sequences in the NCBI Genebank. Most of the isolates in this study were identified with strains of the genus *B. cepacia* while a few others related to the genus *Ralstonia* (Table 2.3). The species identities of the isolated strains are as shown in Table 2.3 and they included six *B. territorii*, six *B. cepacia*, one *B. metalica*, one *B. cenocepacia*, one *Burkholderia sp* and one *Ralstonia pickettii*. Four other bacterial isolates including MZM SPr, KS PTg, KS PT4 and KH Rc were not identified due to low quality and quantinty of PCR product for sequencing.

Figure 2.3 shows the phylogenetic tree including all identified bacterial isolates from this study. Some bacteria showed close similarity in gene sequences obtained from NCBI GenBank. *Burkholderia sp*. QN_m1 was closely related to *R. picketii* strain r505, *B. territorii* strain S2 was closely related to *B. cepacia* strain GYP1 and two strains of *B. territorii* strain KBB5 formed the cluster together which was closely related to *B. cepaciia* strain GYP1. The number beside the node is the statistical bootstrap value. The tree was rooted using *Burkholderia metallica* strain BAB-6777 as an out-group.

Table 2.3: Molecular identification of bacterial isolates based on 16s rRNA gene sequencing

Isolate	Matched bacteria	Similarity	Genebank Accession

		(%)	number
MZM SPr	Unidentified		
KS PTx	*Burkholderia metallica* strain BAB-6777	99.52%	MF319855.1
KS PTg	Unidentified		
KS PTn	*Burkholderia territorii* strain KBB5	99.76	MN032407.1
KS PT5	*Burkholderia cepacia* strain GYP1	99.65	KY697917.1
KS PT4	Unidentified		
SUA R1	*Burkholderia territorii* strain KBB5	99.65	MN032407.1
RF	*Burkholderia cepacia* strain GYP1	99.65	KY697917.1
SUA R3	*Burkholderia cepacia* ATCC 25416	99.4	CP034553.1
KS PT2	*Burkholderia cepacia* ATCC 25416	99.76	CP034553.1
KH Rc	Unidentified		
KH Ra	*Burkholderia cepacia* strain GYP1	99.65	KY697917.1
MZM SPo	*Ralstonia pickettii* strain r505	96.91	MK934373.1
MZM SPk	*Burkholderia cenocepacia* strain YG-3	99.06	CP034546.1
MZM SPh	*Burkholderia sp.* ON_m1	96.76	LC487324.1
KH Rj	*Burkholderia territorii* strain S2	99.25	MN044777.1
MZM R12	*Burkholderia territorii* strain S2	99.72	MN044777.1
KS PTk	*Burkholderia cepacia* strain GYP1	99.18	KY697917.1
SIA R111	*Burkholderia territorii* strain S2	99.51	MN044777.1
MZM R211	*Burkholderia territorii* strain KBB5	99.63	MN032407.1

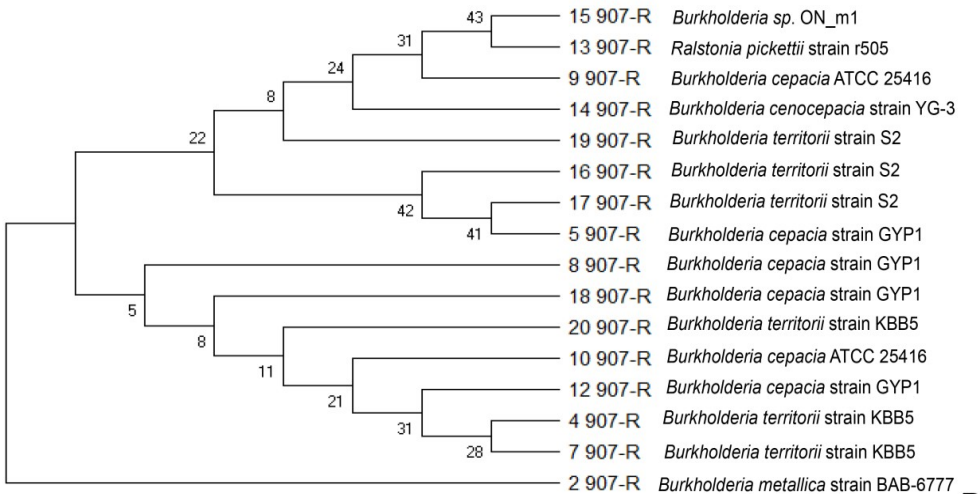

Figure 2.3: Phylogenetic tree based on 16S rNA sequences showing the position of *Burkholderia* species and *Ralstonia pickettii* strains with regard to related species.

2.5 Discussion

In the present study sweet pepper, rice and potato root sysytems (Table 2.1) were choosen for isolation of phosphate solubilizing bacteria due to greater possibility of occurrence of phosphate solubilizing bacteria. Mohammadi (2012) reported that most of the metabolic active phosphate solubilising bacteria are concentrated in the rhizosphere of different crops and interact with crop

root surfaces for nutrients aquisation. Phoshate solubilising bacteria have been isolated from root surfaces of rice (Ji *et al*., 2014; Gopalakrishnan *et al*., 2011), sweet pepper (Alia *et al*., 2013) and potato (Alia *et al*., 2013; Dawwam *et al*., 2013). The variation in the size of clear zone and of blue colour strength (Plate 2.1) was visually considered as variation in phosphate solubilising ability, as reported by Bharucha *et al*. (2013). The varying ability in phosphate solubilisation in both the solid PVK and liquid PVK media (Figures 2.1 and 2.2 respectively) matches the findings by other researchers (Chen *et al*., 2006; Chung *et al*., 2005; Perez *et al*., 2007; Hariprasad and Niranjana, 2009) who also indicated the varying ability of bacterial isolates to solubilise insoluble mineral phosphate compounds. This could probably be due to difference in bacterial genomic and plasmid properties as reported by Bapiri *et al*. (2012).

Phosphate solubilisation index (Fig. 2.1) was not directly proportional to the amount of solubilized phosphate concentration (Fig. 2.2). Bacterial isolates KHRa, SUA R3, MZM SPk and KS PT5 which released high amount of soluble phosphorus were observed to have low phosphate solubilisation index. Similar results have been reported by other authors (Gupta *et al*., 1994; Lynn *et al*., 2013; Nautiyal, 1999). Often qualitative assay is used as preliminary tool for evaluating phosphate solubilisation (Li *et al*., 2017). However, this method relies more on the ability of microorganisms to form a halo zone on a solid medium and it fails to account for phosphate solubilisation especially when the halo zone is inconspicuous or absent (Mehta and Nautiyal, 2001). Thus, these findings suggest that qualitative phosphate solubilisation should not solely be used in evaluating phosphate solubilisation efficiency; instead, quantitative measurement of P solubilizing should be used to get more reliable inferences (Baig *et al*., 2014; Li *et al*., 2017).

As reported by other researchers (Chaiharn and Lumyong, 2009; Charana Walpola and Yoon, 2013; Fankem *et al*., 2006), the amount of soluble P released in the liquid PVK medium (Figure 2.3) was

observed to vary with advanced period of incubation. Production of organic acid during incubation period is believed to be the main mechanism for phosphate solubilisation. Carboxylate group of organic acids chelate the cations (mainly Ca) bound to phosphate thereby converting them into soluble forms (Fankem *et al*., 2006; Kpomblekou and Tabatabai, 1994; Park *et al*., 2010). The observed reductions in rate of release of soluble phosphorous during the later stages of the incubation might be due to the depletion of nutrients in the culture medium, in particular, carbon source needed for the production of organic acids which resulted either to decrease in the phosphate solubilisation efficiency or the number of phosphate solubilising bacteria in the medium (Kang *et al*., 2002; Chaiharn and Lumyong, 2009). Also production of toxic excretory products and refixation of soluble P by metallic ions could be additional possible reasons for reduction in phosphate solubilisation during the later stages of the incubation (Gaur, 1990; Illmer and Schinner, 1992; Patel and Parmar, 2013). Deepa *et al*. (2010) suggested that the decrease in P content with the advance of incubation period could be due to the utilization of soluble phosphorus by bacterial species resulting in the fluctuating levels of P release. The findings of this study further showed that bacteria-crop interaction can lead into differences in phosphate solubilisation efficiency among similar strains. This has also been reported by some other reasearchers (Luvizotto *et al*., 2010; Javadi *et al*., 2015) who suggested that it could be due to genomic diversity.

The existence of common morphological features among bacterial isolates (Table 2.2) indicated that these bacteria could be species of the same genera as reported by Linu *et al*. (2009) stated that bacteria belonging to the same genera tend to have related features and similar gram staining reactions. Both *Burkholderia* and *Ralstonia species* which were the genera identified in this study (Table 2.3) have been reported as efficient phosphate solubilisers (Azziz *et al*., 2012; Kailasan and Vamanrao, 2015; Khalimi *et al*., 2012; Midekssa *et al*., 2016; Pei-Xiang *et al*., 2012; Viruel *et al*., 2011; Silini-Cherif, 2012). The observed phylogenetic close relationship between bacteria of genus

Burkholderia (Figure 2.3) is not surprising since the genus *Burkholderia* is among the most diverse genera and is believed to contain over 30 species occupying remarkably diverse ecological niches including rhizosphere of different plants (Coenye and Vendome, 2003; Dalmastri *et al.*, 2005). Close relationship observed between *Ralstonia picketii* strain r505 and *Burkholderia sp*. QN_m1 (Figure 2.3) are not surprising since other authors (Eberl and Vandamme, 2016; Peddayelachagiri *et al*., 2016; Ramette *et al*., 2005; Voronina *et al*., 2015) reported that some species of *Ralstonia* were once included in the genus *Burkholderia*. Generally close similarity in DNA sequence of these bacteria suggests that they could have originated from the same ancestor. However, there is weak evidence to support that closely related bacteria are the same microorganism as evidenced by low bootstrap support (< 97%).

Phosphate solubilising bacteria improve phosphorus nutrition, as a result enhances plant growth and yield (Hariprasad and Niranjana, 2009; Yazdan *et al*., 2009). In the present study, native isolates *Burkholderia cepacia* strain GPY1, *Burkholderia cepacia* strain ATCC 25416 and *Burkholderia cenocepacia* strain YG3 were found to be the most efficient phosphate solubilising strains as compared to other strains: this gives clue for the future use the strains in improving phosphorus nutrition and enhancing plant growth.

2.6 Conclusion

In this study, different species of genus *Burkholderia* were observed to have varying ability to solubilise insoluble $Ca_3(PO_4)2$. The potential ability to solubilise insoluble phosphate exhibited by *Burkholderia* species, implies their potential for use in enhancing phosphorus availability tor plants. All isolates indicated promising ability to solubilise Ca3(PO4)2 under controlled environment. However, *Burkholderia cepacia* strains were observed to be most efficient in phosphate solubilisation. Thus, these strains need to be evaluated for their efficiency under other conditions,

especially field trials so as to confirm these findings before recommending the strains for commercial applications.

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CHAPTER THREE

3.0 PLANT GROWTH PROMOTING POTENTIAL OF BACTERIA ISOLATED FROM ROOT SURFACES OF SELECTED FIELD AND GARDEN CROPS AROUND MOROGORO MUNICIPALITY, TANZANIA

Abstract

Burkholderia and *Ralstonia* species are among the known plant growth promoting rhizobacteria (PGPR) used to enhance plant growth through various mechanisms, including nutrient solubilisation and production of various phytohormones and biocontrol agents. This study reports on zinc solubilisation, indole-3-actetic acid (IAA) and siderophore production by bacterial species of *Burkholderia* and *Ralstonia* and effects of their direct application on growth of maize (*Zea mays* L.) under laboratory and screen house conditions. The study was arranged in triplicates in the Completely Randomized Design (CRD). Raw data were subjected to analysis of variance (ANOVA) using the GenStat Discovery 15th edition software, and treatment means were ranked using the Duncan's Multiple Range Test at 5% probability (P = 0.05). There was significant (P = 0.05) variation between bacterial groups. The maximum amount of IAA was 28 mg L^{-1} produced by bacterial isolates *Burkholderia cepacia* strain ATCC 25416 followed by *Burkholderia cepacia* strain GYP1 which produced 21 mg L^{-1}. All bacteria were efficient siderophore producers. *Burkholderia sp*. QN m1 indicated the maximum percentage siderophore unit (PSU) 95% followed by *Burkholderia territorii* strain KBB5 (94.82%) and *Burkholderia territorii* strain S2 (93.98%). On the other hand, the maximum soluble zinc was 347.5 mg L^{-1}, released by *Burkholderia territorii* strain KBB5, followed by *Burkholderia cepacia* strain ATCC 25416. Results on plant growth indicated that inoculating bacteria on growing plantlets increased maize growth. There was significant increase (P = 0.05) in both plant height and root length in some bacterial treated plants as

compared to control. The maximum root lengths under laboratory conditions ranged from 1.2 cm in control to maximum 7.4 cm observed for *Burkholderia cepacia* strain GYP1 isolated from Irish potato. Plant height on the other hand ranged from the lowest length, 1.2 cm, observed in control plants to a maximum 10.3 cm observed for *Burkholderia cepacia* strain GYP1 isolated from Irish potato. Results under screen house experiment indicated that root elongation ranged from a minimum 28.5 cm in water treated plants to a maximum 68 cm in plants treated with *Burkholderia cepacia* strain GPY1. On the other hand, plant height ranged from 46.8 cm in water treated controls to a maximum 81.3 cm in plants treated with *Burkholderia cepacia* strain ATCC 25416 and *Burkholderia cepacia* strain GYP1. Findings of the present work indicate that these bacteria isolates can effectively be used as plant growth promoters, as they can significantly increase plant growth. However, this study recommends further research on the effectiveness of the inoculant under uncontrolled conditions like field trials before use for commercial purpose.

Key words: Indole-3-acetic acid (AA), Plant growth promoting rhizobacteria (PGPR), Maize (Z*ea mayse* L), Phosphate solubilisation, Siderophore production, Zinc solubilisation.

3.1 Introduction

A group of bacteria colonizing the rhizosphere, known as plant growth promoting rhizobacteria (PGPR), have potential ability to enhance plant growth (Ibiene *et al*., 2012) and improve plant health and soil fertility (Ahmad *et al*., 2008). PGPR enhances plant growth through various mechanisms, directly or indirectly. Direct mechanisms include production of plant hormones, nitrogen fixation, and solubilisation of nutrients (Ahmad *et al*., 2008). Indirect mechanisms include antagonism against phytopathogenic microorganisms by the production of siderophores, synthesis of antibiotics, enzymes or fungicidal compounds or competition with harmful microorganisms (Ahmad *et al*., 2008; Senthilkumar *et al*., 2009). Production of phytohormones such as Indole-3-acteic acid (IAA) allows plants to develop longer roots and better establish during early stages of growth (Ahmad *et al*., 2008; Marques *et al*., 2010). PGPR inoculants have been used in agriculture to improve soil fertility by increasing the level of plant available nutrients such as phosphorus, potassium and zinc (Ahmad *et al*., 2008). Also, application of PGPR has been reported to reduce the frequent use of agrochemicals including fertilisers, fungicides and other pesticides which have a tendency to pollute the environment and contaminate soils (Gupta *et al*., 2015). In the present study bacterial isolates belonging to the genera *Burkholderia* and *Ralstonia*, which were observed to solubilise insoluble phosphate (tri-calcium phosphate) as demonstrated in previous chapter (Chapter 2), were evaluated for their plant - growth promoting abilities including zinc solubilisation, indoleacetic-3-acid (IAA) and siderophore production and their effect on maize growth.

3.2 Materials and Methods

3.2.1 Location of study area

Both laboratory and potted soil experiment were conducted within the laboratories and screen houses of the Department of Soil and Geological Sciences of Sokoine University of Agriculture (SUA), respectively.

The screen house is located within main campus of Sokoine University of Agriculture, at the Latitude 06º51’ S and longitude 37º39 E and an elevation of 550 m above the mean sea level.

3.2.2 Bacterial inoculants used in the study

A total of 20 bacterial types were isolated from root surfaces of field and garden crops around Morogoro, Tanzania. The isolates were molecularly identified (as detailed in chapter two) and they included six species of *B. territorii* and *B. cepacia*, one *B. metalica*, one *B. cenocepacia*, one *Burkholderia sp* and one *Ralstonia pickettii*. Other four isolates were not identified due to low quality and quantity of PCR products for sequencing.

3.2.3 Zinc solubilisation assay

Zinc solubilisation was evaluated using minimal salt agar and liquid media for qualitative and quantitative solubilisation assay. Mineral salt agar contained 10 g dextrose, 15 g agar, 1 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g K_2HPO_4, 0.2 g $MgSO_4$ and 1 g insoluble $ZnCO_3$ into 1000 mL distilled water, and the final pH was adjusted to 7.0 (Gandhi, 2016). Qualitative zinc solubilisation was carried out as described by Fasim *et al*. (2002), whereby loopful of a single colony of each purified isolate was spot-inoculated at the centre of solidified agar medium in a petridish and incubated upside-down at 28 ^oC for ten days. The study was arranged in triplicates following the Completely Randomized Design (CRD). Diameters of clear zones and their corresponding colonies were measured on the 3rd, 5th and 9th days of incubation using a sterile meter rule, and the zinc solubilisation index was calculated according to Liu *et al*. (2015):

$$ZSI = \frac{Colony\ diameter + Halo\ zone\ diameter}{Colony\ diameter}$$

incubator at 150 rpm and 28 °C for 4 days. The amount of soluble zinc released was quantified on the 24, 48, 72 and 96 hours according to the procedure described by Fasim *et al*. (2002). About 15 mL from each culture was withdrawn on the 24 h, 48 h, 72 h and 96 h and centrifuged at 10,000 rpm for 10 min. Four mL of cell free supernatant solution was directly aspirated into an atomic absorption spectrophotometer (AAS) at 213.9 nm wavelength for determination of soluble zinc. Absorbance readings obtained from the AAS were finally converted into soluble zinc concentration using a standard curve prepared from standard of zinc.

3.2.4 Siderophore production assay

Chrome Azurol S (CAS) agar medium was used for the detection of siderophore production. The medium was prepared according to the formulation described by Schwyn and Neilands (1987). Initially, isolates were evaluated for siderophore production onto CAS agar medium modified by including nutrient agar. Solidified CAS agar media in a petridish were cut into two equal halves;, one half was removed and plates were filled by equal amount of nutrient agar. Purified colony of each isolate was streaked onto the nutrient agar medium near to the Chrome azurol S (CAS) agar medium and the plates were incubated at 28 ^{o}C for 48 to 72 h. Bacterial isolates forming yellow-orange colour on a CAS medium adjacent to the growing colony (on nutrient agar) were regarded as siderophore producers (Ahmad *et al*., 2008) and were further studied quantitatively. Quantitative estimation of siderophore production was done using a succinate broth which was prepared by mixing (g L^{-1}) 6 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$ and 4 g succinic acid. The pH of the medium was adjusted to 7 using 0.5 N NaOH (Sharma and Johri, 2003). A loopful of each purified (siderophore producing) bacterial culture was aseptically transferred into 50 mL of previously sterilised succinate acid medium contained in a 100 mL sterile bottle and incubated at 28 ± 2°C with continuous shaking at 180 rpm for 24 h. The experiment was arranged in the completely randomized design (CRD) with three replicates. About 20 mL from each 24 h old bacterial culture

were centrifuged at 10,000 rpm for 10 min, cell pellets were discarded and the supernatant solution was used to estimate siderophore according to the procedure described by Radzki *et al*. (2013). 0.5 mL of the supernatant solution from each bacterial culture were mixed with 0.5 mL of CAS reagent and allowed to stand for 20 min for colour development. The intensity of the developed yellow-orange colour was measured at 630 nm wavelength using a spectrophotometer. Absorbance of reference was taken by including a control, which was prepared by mixing a CAS reagent and a bacteria-free succinate medium. Siderophore produced by strains was estimated as the percentage of siderophores unit (PSU), which was calculated according to Pai and Gokarn (2010) as:

$$PSU = \frac{Ar - As}{Ar} \text{ x } 100$$

where Ar is the absorbance of reference (CAS reagent and uninoculaed, culture-free succinate medium), As is the absorbance reading from a sample.

3.2.5 Indole-3-acetic acid (IAA) production assay

Bacterial cultures were inoculated into 50 mL of sterilised nutrient broth (NB) supplemented with 50 mg tryptophan (Gopalakrishnan *et al*., 2015). After inoculation, each culture was incubated at 28 ± 2°C, with continuous shaking at 180 rpm for 24 h. The experiment was arranged in the completely randomized design (CRD) with three replicates. About 15 mL of each culture was centrifuged at 10, 000 rpm for 10 minutes and the cell-free supernatant solution was used in IAA quantification based on the method by Madhurama *et al*. (2014). 1 mL aliquot of the supernatant was mixed with 2 mL of Salkowski's reagent and incubated for 20 min in darkness at room temperature. IAA production was observed as the development of a pink-red colour (Ahmad *et al*., 2008), and the absorbance was measured at 530 nm using a spectrophotometer. The concentration of IAA was determined using a standard curve prepared from pure IAA solutions (0, 5, 10, 20 and 50 µg mL^{-1}).

3.2.6 Preparation of maize seeds for inoculation and bacteria inoculants

Hybrid maize variety Seed.CO (SC 430) was used as host plant in this experiment. Seeds were surface sterilised by soaking in 95% ethanol for 30 seconds twice, followed by rinsing with sterile distilled water ten times after each soaking.

Bacterial inoculants were prepared using nutrient broth (NB) medium suplimented with L-tryptophan at the rate of 1 mg mL^{-1} (Gopalakrishnan *et al*., 2015). A loopful of each pure bacterial culture was inoculated into previouslty sterilized nutrient broth medium containing L-tryptophan and left to grow for 24 h at room temperature on a shaking incubator at 180 rpm.

3.2.7 Determination of the effect of bacterial inoculation on maize plant growth

Two sets of experiments were conducted to evaluate the potential effects of bacterial inoculation on plant growth, one being under laboratory conditions and the other under screenhouse conditions.

For the laboratory experiment, petridishes were washed with distilled water and air-dried before lining them with two layers of filter papers. The lined petridishes were then autoclaved at 15 psi (103.4 kPa) pressure, 121^{o}C temperature for 15 minutes. After cooling, two sterile maize seeds were aseptically positioned into each petridish and 10 mL of 24 h old bacterial culture were applied into respective petridishes. A control treatment contained seeds and 10 mL of sterile medium without bacterial inoculum. The experiment was arranged according to the completely randomized design (CRD) with two replications. Plantlets were allowed to grow for 13 days after which they were harvested and growth parameters (root length and shoot height) were recorded.

On the screenhouse based experiments, soils were sterilised by autoclaving at 15 psi pressure, and 121^{o}C temperature for 15 minutes. Pots were previously washed with tap water followed by soaking in 95% ethanol for 10 minutes and then rinsed with sterile distilled water 5 times. Four kg sterile soil was then filled into sterile pots and four previously surface sterilized maize seeds were sown in

each pot at equal depth, approximately 1 cm depth each. Thinning was done at the fifth day and two plantlets in each pot were allowed to grow for 30 days. Inoculation was done on the sixth day by pouring 20 mL of overnight bacterial culture grown in nutrient broth (NB) medium supplemented with 20 mg of L-tryptophan. A control pot was treated with sterile medium without bacterial inoculant. Soils in each treatment were moistened with an equal volume of autoclaved distilled water after every other day. The treatments were arranged in the completely randomized design with two replications. Plantlets were allowed to grow for 30 days and harvested. Plant growth parameters, i.e. root length and shoot height for each treatment, were recorded.

3.3 Data Analysis

All raw data generated during the experiments were subjected to analysis of variance (ANOVA) using the GenStat Discovery 15th edition software, and treatment means were ranked using the Duncan's Multiple Range Test at 5% probability (P = 0.05).

3.4 Results

3.4.1 Zinc solubilisation

Formation of clear zone on the mineral salt agar plate supplemented with insoluble $ZnCO_3$ (Plate 3.1) indicated zinc solubilisation activities (Sharma *et al*., 2011). There were significant (P = 0.05) variations in ability to solubilize insoluble zinc resources. Minimum zinc solubilisation index (ZSI) was 2.701 attained on the third day by isolate MZM SPr (unidentified). Zinc solubilisation index (ZSI) values for each bacterium increased with advanced incubation period and the maximum ZSI value was 7.056 attained by isolate KS PT4 (unidentified) on the seventh day of incubation as depicted in Figure 3.1. The amount of soluble zinc released by bacterial isolates also increased with advanced incubation period. However, further incubation showed the decrease in soluble zinc concentrations in some isolates. Generally, *Burkholderia territorii* strainKBB5 isolated from potato was noted as the most potent strain, solubilising the highest amount of zinc, 347.5 mg L^{-1} and 334.85 mg L^{-1} on 72 and 96 h, respectively, as depicted in figure 3.2.

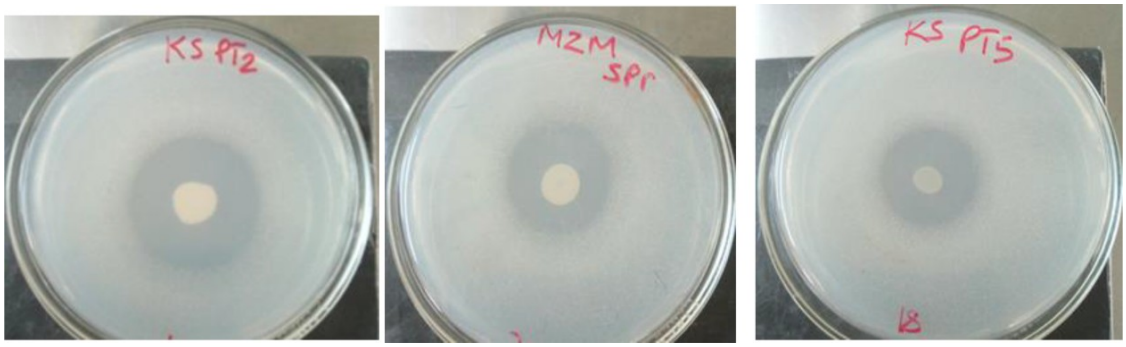

Plate 3.1: Presence of clear zone on a mineral salt medium supplemented with insoluble zinc carbonate, indicating zinc solubilisation

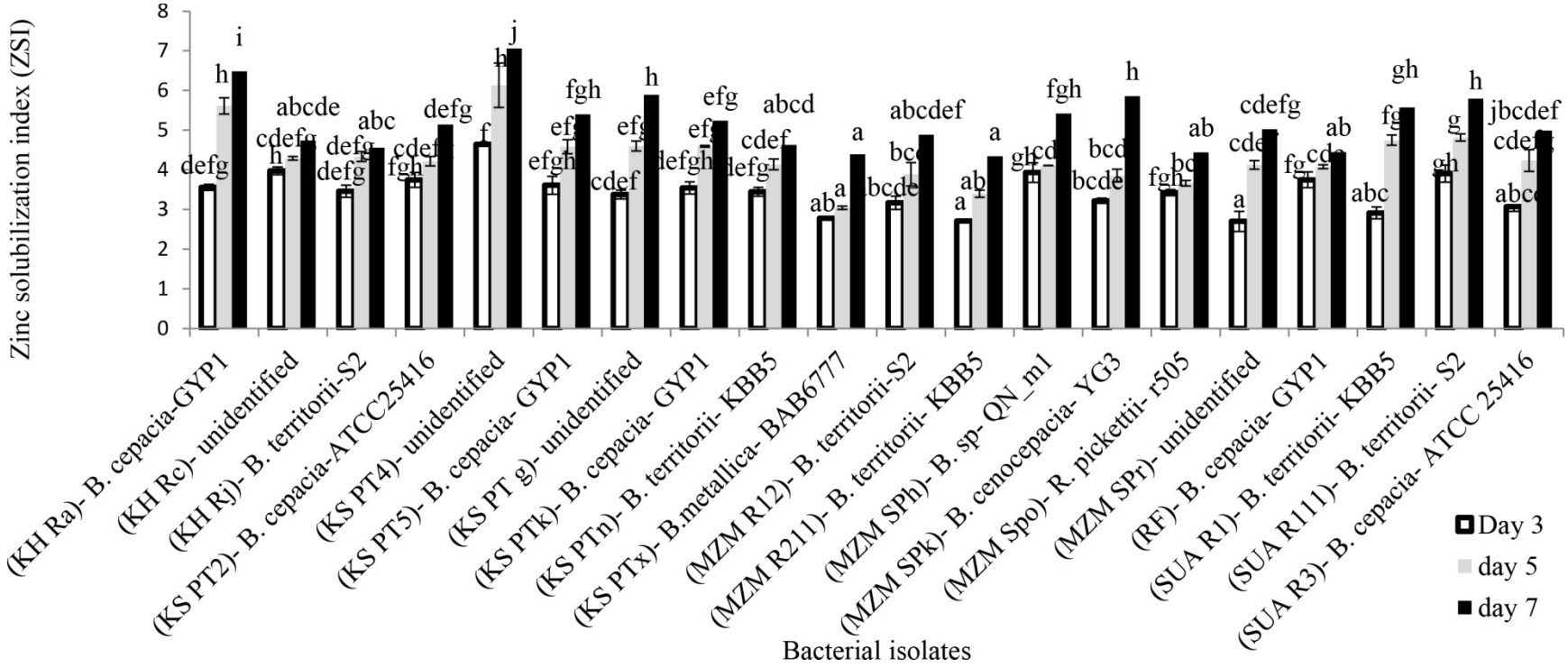

Figure 3.1: Zinc solubilisation index values for bacterial isolates on a mineral salt agar plate supplemented with insoluble $ZnCO_3$.

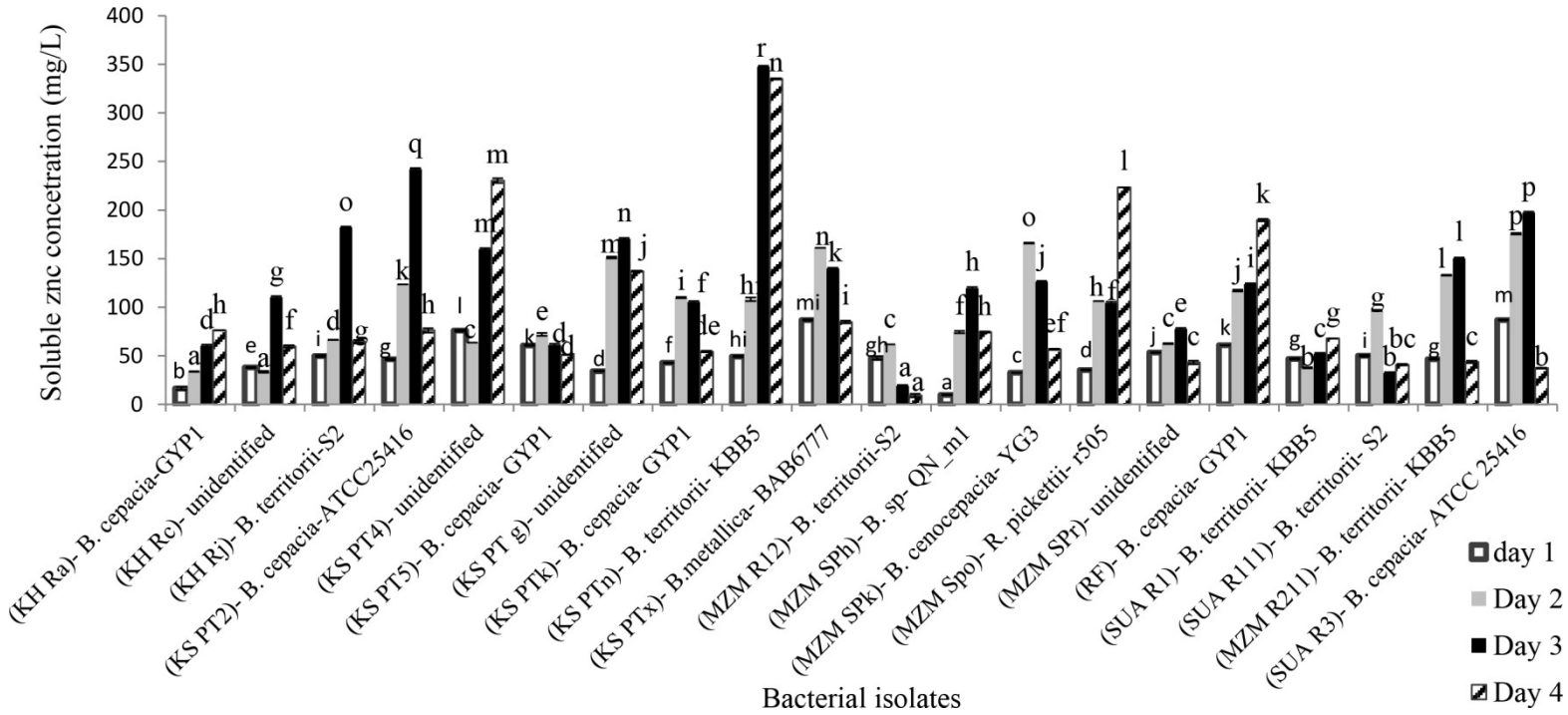

Figure 3.2: Quantitative zinc solubilisation by various bacterial isolates in mineral salt- $ZnCO_3$ liquid medium.

3.4.2 Siderophore production

The yellow-orange colour formed on solid CAS medium and aliquot supernatant solution (added with CAS reagent) (Plate 3.2) indicated siderophore production (Ahmad *et al*., 2008). The amount of siderophore produced by bacterial isolates as quantified in percentage siderophore units (PSU) significantly (P = 0.05) varied among bacterial strains (Figure, 3.3). *Burkholderia sp*. QN m1 indicated slightly higher siderophore percentage unit (95%), which was not statistically (P = 0.05) different from *Burkholderia territorii* strain KBB5 (94.82%) and *Burkholderia territorii* strain S2 (93.98%). On the other hand, the lowest qunatinty of siderophore was produced by bacterial isolate *Burkholderia cepacia* strain GYP1 (28.77%), as depicted in Fig. 3.3.

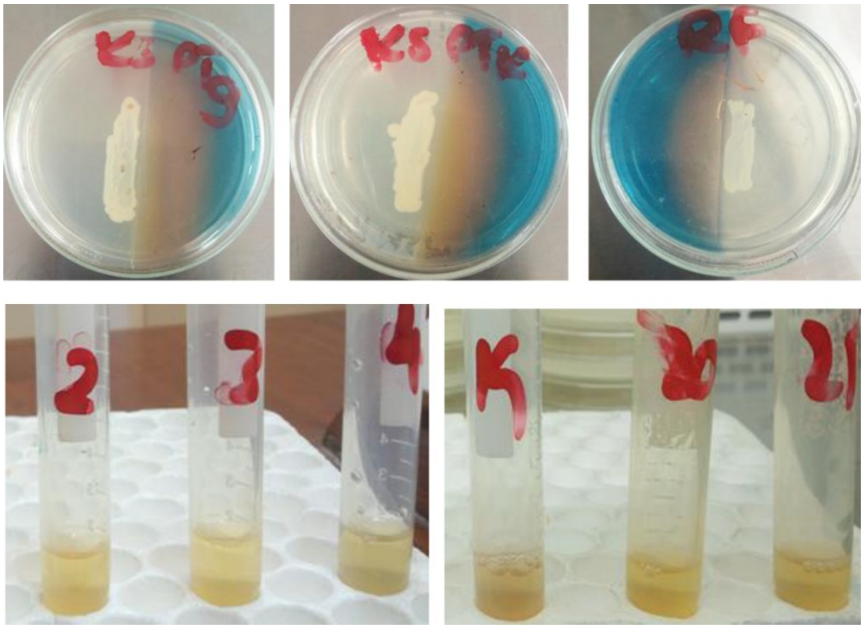

Plate 3.2: Top: Presence of yellow-organ colour on a modified nutrient agar medium and aliquot supernatatnt solution (top and bottom, respectively) added with CAS reagent as an indicator for siderophore production by bacterial isolates.

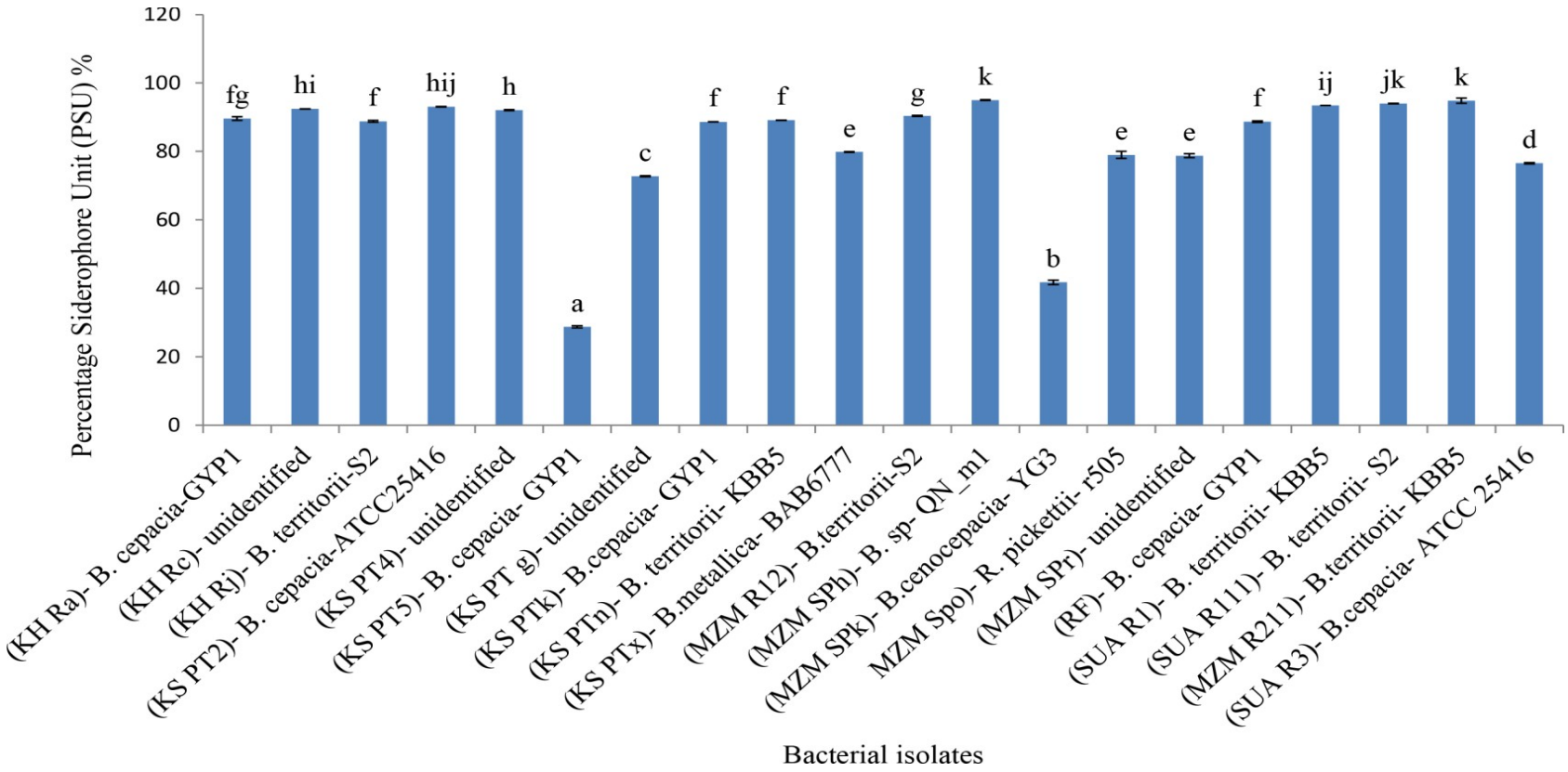

Figure 3.3: Percentage siderophore unit values by various bacterial isolates in succinate liquid medium.

3.4.3 IAA production

Results on IAA production by different isolates are presented in Figure 3.4. Amounts of IAA produced significantly (P = 0.05) varied among bacterial strains. Also, similar strains isolated from different crop sources indicated different efficiencies in IAA production. The maximum amount of IAA was 28.761 mg L^{-1}, produced by *Burkholderia cepacia* strain ATCC 25416 isolated from rice root surface, followed by Burkholderia *cepacia* strain GPY1 isolated from potato root surface. The lowest amount of IAA was 1.286 mg L^{-1}, which was produced by *Burkholderia sp.* QN m1 isolated from sweet pepper (Figure 3.4).

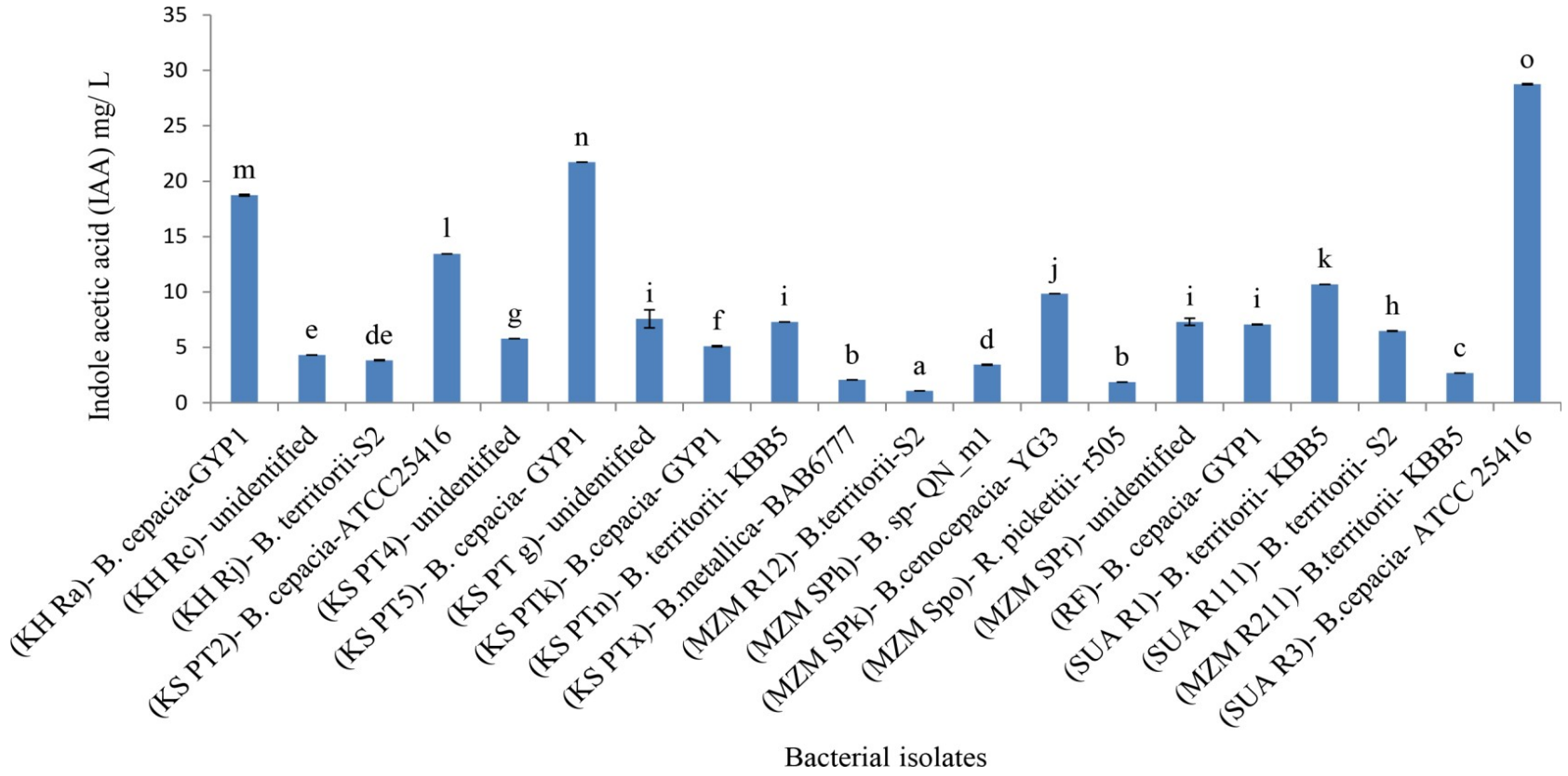

Figure 3.4: Quantitative indole acetic acid (IAA) producton by various bacterial isolates in nutient broth supplemented with L-tryptophan.

3.4.4 Effects of bacterial isolate innoculations on plant growth promotion

3.4.4.1 Laboratory experiment for corn growth promotion

All bacterial isolates were observed to positively influence root and shoot elongation (Plate 3.3) with some strains showing startistically significant (P = 0.05) effect when compared to water or medium-only treated control plants. Root elongation varied from a minimum of 1.2 cm (observed in the control group) to a maximum value of 7.4 cm (observed in plants inoculated by *Burkholderia cepacia* strain GPY1 isolated from Irish potato). Other strains including *Burkholderia territorii* strain S2, *Ralstonia pickettii* strain r505, *Burkholderia territorii* strain S2 and *Burkholderia metallica* strain BAB-6777 were observed to have little effects on root length, which were not significantly (P = 0.05) different as compared to medium-only treated control plants (Figure 3.5). Bacterial isolates *Burkholderia* sp. QN m1, *Burkholderia territorii* strain S2, B*urkholderia territorii* strain S2 and *Ralstonia pickettii* strain r505 did not significantly (P = 0.05) promote plant height as compared to the control while the rest were observed to promte shoot elongation significantly (P = 0.05) (Fig. 3.6). Generally, shoot elongation ranged from a minimum length of 1.2 cm observed in control plants to a maximum of 10.3 cm observed in plants inoculated bwith *Burkholderia cepacia* strain GPY1.

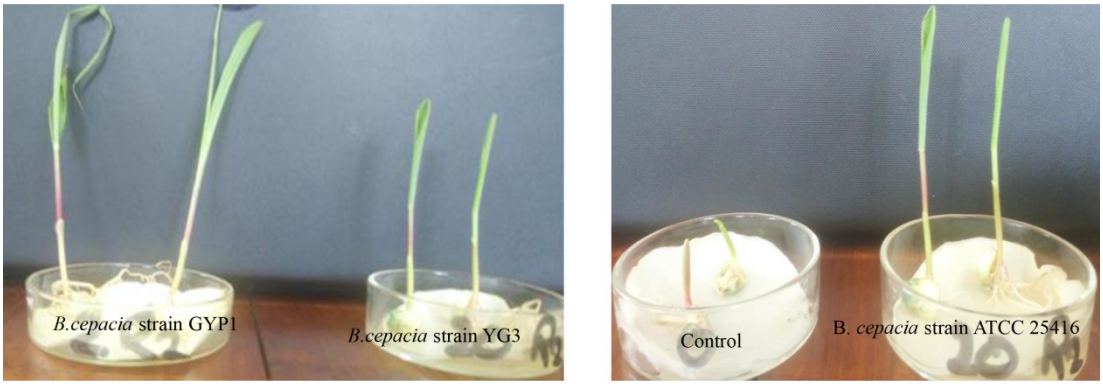

Plate 3.3: Maize response to bacterial inoculation as was observed at a 10th day *B. cepacia* strain GYP1 inoculation resulted into high plant heigh, followed by *B cepacuia* strain ATCC25416 and *B. cepacia* strain YG3. The lowest plant heigh was observed in a control plate which contained medium without bacterial inoculum.

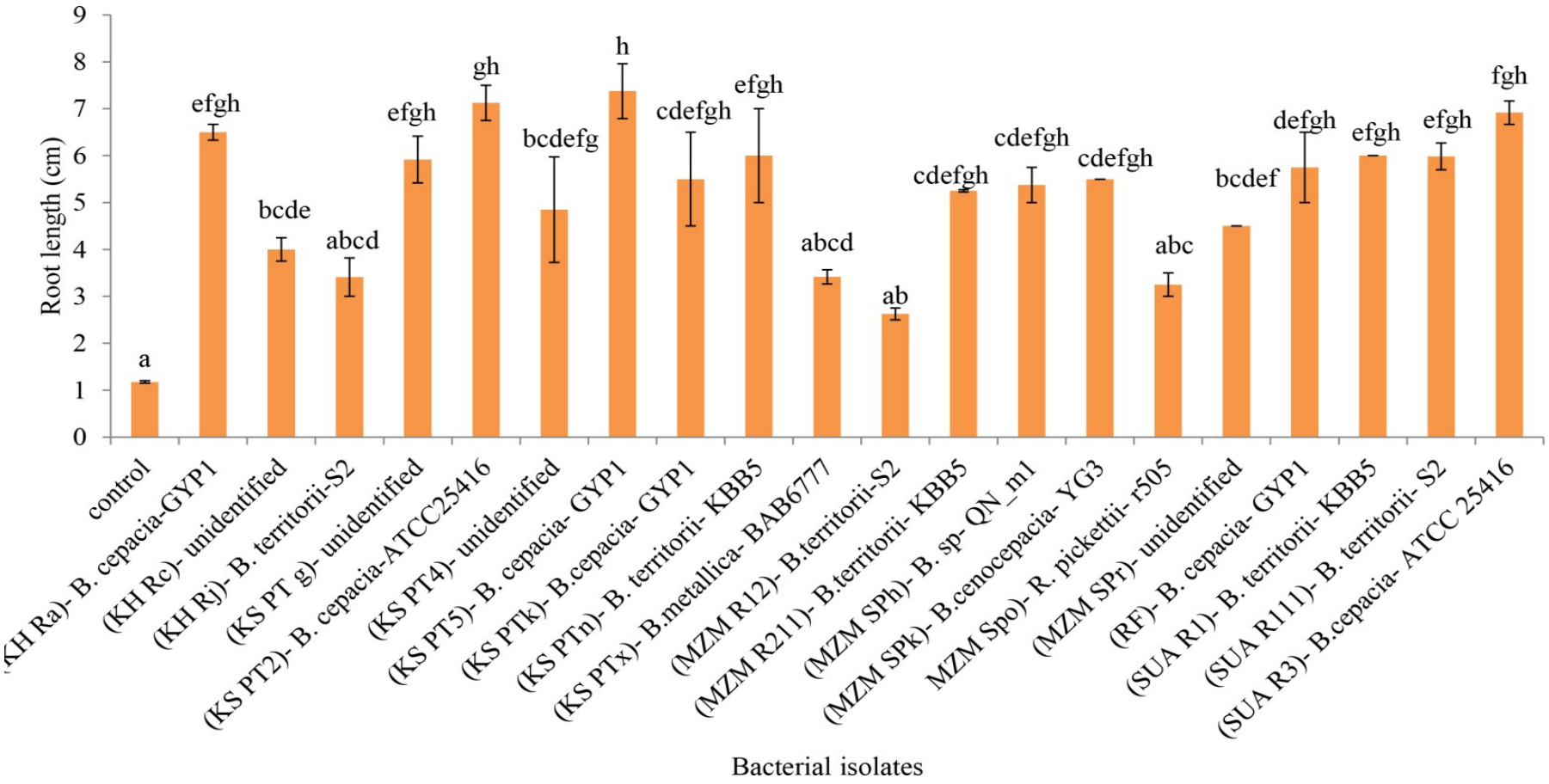

Figure 3.5: Effect of bacterial inoculation on maize root elongation. Seeds were inoculated by overnight (24 h) old bacteria grown into nutrient broth supplemented with tryptophan.

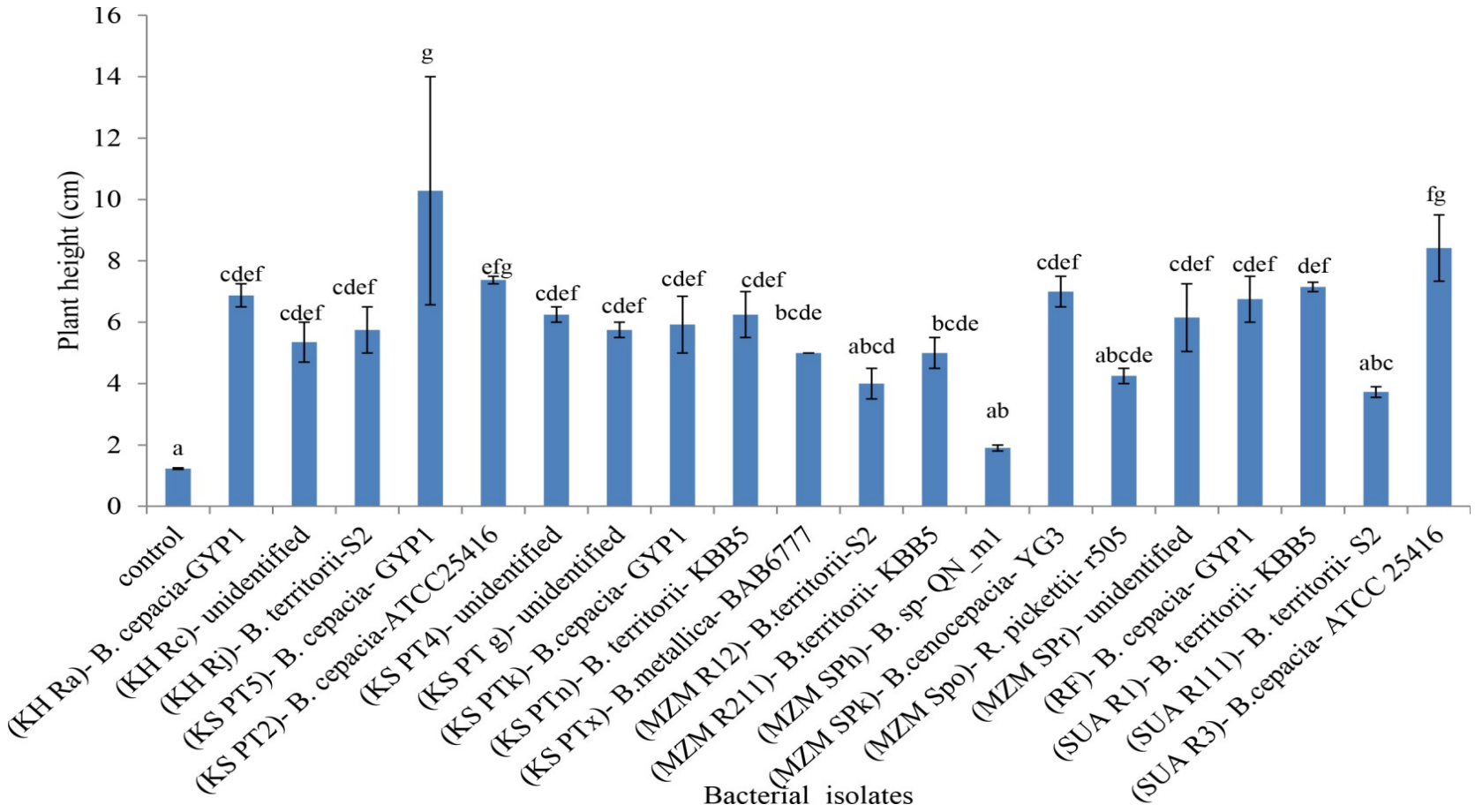

Figure 3.6: Effect of bacterial inoculation on maize height.

3.4.4.2 Screen house experiment for maize growth promotion experiment

Plate 3.4 shows representative plants and roots for bacterial inoculated and uninoculated controls. The maximum root length was 68 cm registered in plants inoculated with the bacterial isolates *Burkholderia cepacia* strain GPY1isolated from rice root surface followed by *Burkholderia cepacia* strain GYP1 (63.75 cm) isolated from potato and *Burkholderia cepacia* strain ATCC 25416 from rice (63.5 cm). The control plants showed the lowest root elongation of 28.5 cm which was not significantly (P = 0.05) different from root lengths observed in plants inoculated with isolates KS PT4 (unidentified) and *Burkholderia territorii* strain KBB5, as depicted in Figure 3.7. On the other hand, all bacteria indicated significant (P = 0.05) effects on plant height when compared to control plants. The minimum plant height was 46.75 cm, observed in control plants, while the maximum plant height was 81.25 cm observed in plants treated with *Burkholderia cepacia* strain ATCC 25416 and *Burkholderia cepacia* strain GYP1(Figure 3.8).

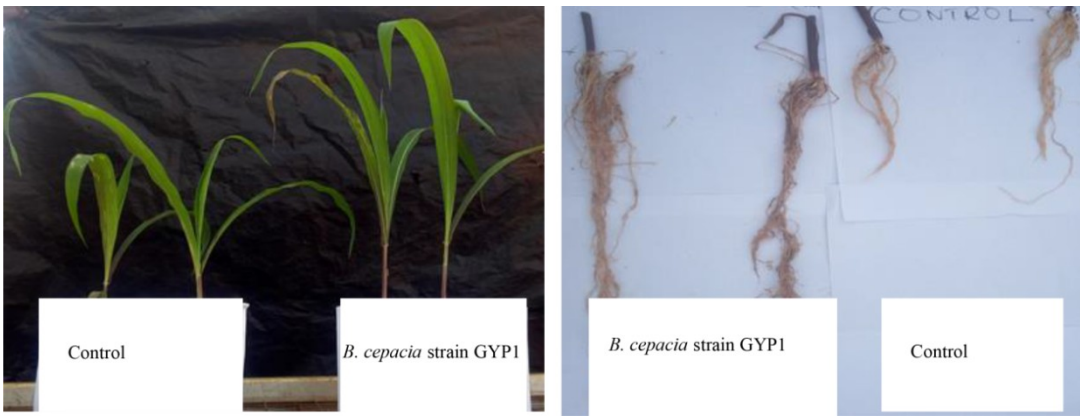

Plate 3.4: Effect of bacterial inoculation on height and root elongation in maize plant observed after 30 days. Plants inoculated with *B. cepacia* strain GYP1 demonstrated high growth promotionon roots and shoots compared to control plants which were treated with bacteria free medium.

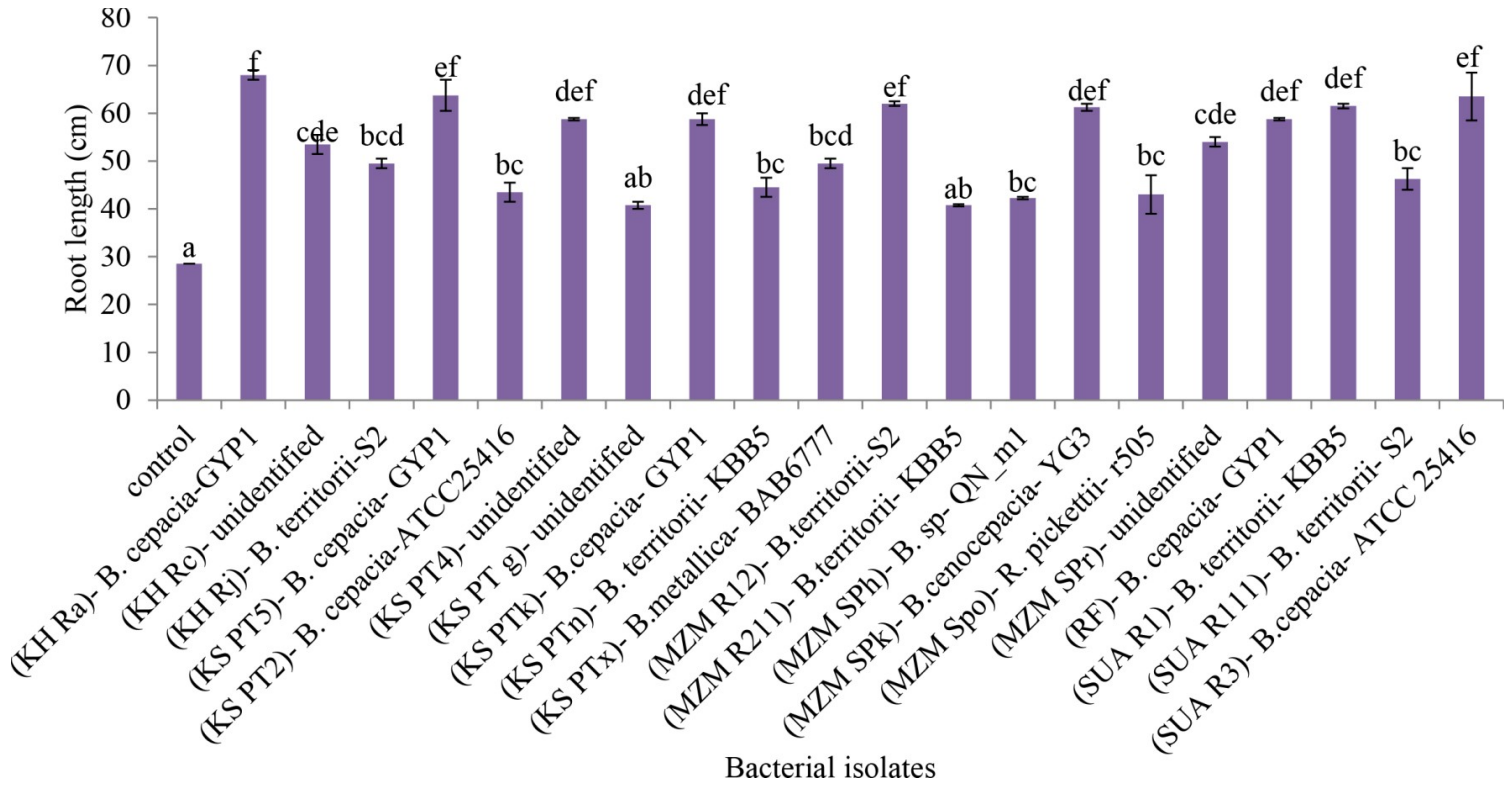

Figure 3.7: Effect of bacterial inoculation on maize root elongation.

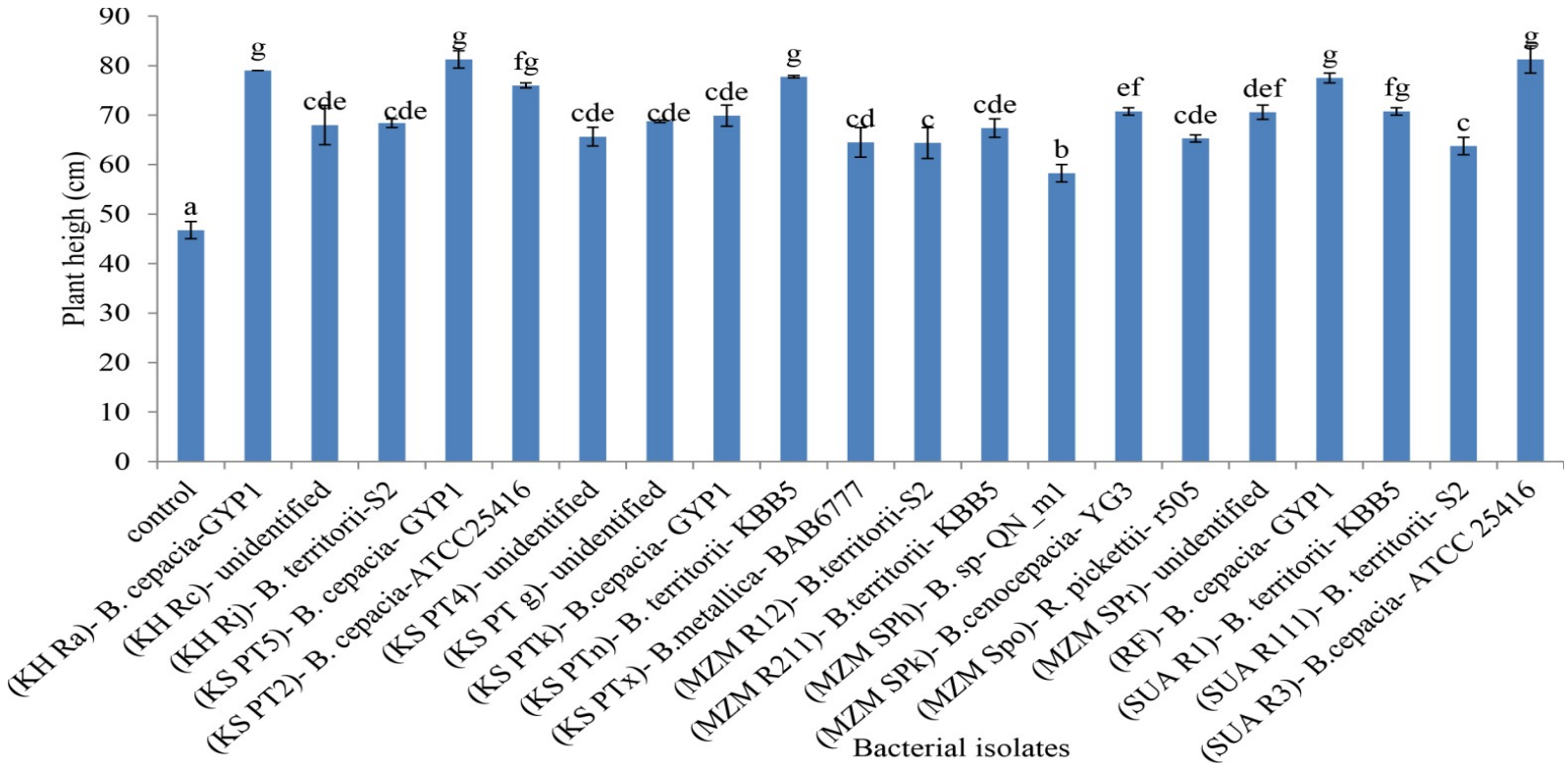

Figure 3.8: Effect of bacterial inoculation on maize height. Overnight (24 h old) bacteria inoculum grown in a NB medium supplemented with tryptophan were poured around emerging plantlets once at a 6th day. Plant heights (cm) were measured after 30 days. Bars carrying different letters or combination of letters are significantly (P = 0.05) different from one another according to the Duncan's New Multiple Range Test

3.5 Discussion

Zinc is an essential element involved in many physiological and metabolic activities of plants, humans and microorganism (Broadley *et al*., 2007). Zinc solubilisation on solid medium as indicated by the formation of clear zone in a solid medium supplemented with insoluble zinc forms reflects zinc solubilisation (Plate 3.1). These observations comply with finings of several other researchers (Fasim *et al*., 2002; Goteti *et al*., 2013; Saravanan *et al*., 2007; Sharma *et al*., 2011) who reported on zinc solubilisation by bacterial isolates. Ability of *Burkholderia* and *Ralstonia* species to solubilise insoluble zinc carbonate complies with the findings of other researchers (Costerousse *et al*., 2010; Dinesh *et al*., 2018; Tagele *et al*., 2018; 2019) who reported on the potential ability of *Burkholderia* species to solubilise insoluble $ZnCO_3$. Solubilisation of insoluble Zn forms by species of *Ralstonia* has been reported in different studies (Gontia-Mishra *et al*., 2017; Jaivel *et al*., 2017). The difference in zinc solubilisation (Figures 3 and 4) could be due to genomic differences. Zinc solubilisation is achieved through several mechanisms (Goteti *et al*., 2013); however, according to Kumar *et al*. (2017), production of organic acids is the major mechanism for zinc solubilisation. The decrease in the pH of the medium with advanced incubation period could be a possible reason for the increase in the concentration of soluble zinc, as reported by in other works (Fasim *et al*., 2012; Sirohi *et al*., 2015; Shruthi, 2013). Also, the build-up of acidic and other toxic constituents during the incubation period have been reported to decrease zinc solubilisation efficiency (Kumar *et al*., 2017).

Formation of yellow-orange colouration (Plate 3.2) signified bacterial ability to produce siderophore (Arora and Verma, 2017). The CAS assay was used since it is the universal assay for siderophore detection and is based on a siderophore's high affinity for ferric iron. Siderophore strongly competes and complexes with ferric iron bound to Chrome azurol S (CAS) medium/dye, resulting in the change of initial blue colour of the Chrome azurol S

(CAS) to the yellow-orange colour (Christina Jenifer *et al*., 2015). Despite that formation of yellow-orange colour on CAS medium/solution is used as a preliminary method for testing siderophore production, the method gives only a rough idea and is not a perfect method for quantification of siderophore production (Arora and Verma, 2017). All bacterial isolates were strong siderophore producers (Figure 3.5). Similar results were obtained by Haas *et al*. (2015); Tagele *et al*. (2019) and Asghar *et al*. (2011) who reported on siderophore production by *Burkholderia* species. Studie done by Bhatt and Denny (2004) and Münzinger *et al*. (1999) also reported on siderophore production by *Ralstonia* species. Siderophore production is very important in enhancing plant growth owing to its role in enhancing iron availability (Bellenger *et al*., 2008; Braud *et al,* 2009) and biocontrol activities (Ren *et al*., 2005).

Bacteria showed varying ability to produce IAA (Fig. 8), results which were similar to those reported by other researchers (Beneduzi *et al*., 2008 Idris, *et al*., 2004; Kumar *et al*., 2012). The observed variations in IAA production beweetn species and strains could be due to differences in physiological conditions of their growth environments, growth stage of cultures at inoculation, and substrate availability for IAA production (Mirza *et al*., 2001; Mohite, 2013). IAA is the most important phytohormone and function as signal molecule in the regulation of plant development (Veerapagu *et al*., 2018). It is well known that IAA stimulates a rapid response (increased cell elongation) as well as a long-term response (cell division and differentiation) in plants (Ahmad *et al*., 2008). Moreover, IAA improves root architecture; for example, it stimulates lateral root formation which, in turn, could provide a high root surface area for nutrient absorption from soil (Compant *et al*., 2010; Glick, 2010). Bacteria belonging to the genus *Burkholderia cepacia* were the most promising IAA producers in the current study.

Both laboratory and screenhouse experiments were conducted under controlled conditions to eliminate variations due to external factors. The same potting media were used, and all pots were uniformly moistened while screenhouse conditions were controlled to minimize seasonal temperature changes. Thus, it is worth to conclude that the observed variations were mostly due to microbial activities. High growth of maize plants inoculated with *Burkholderia* and *Ralstonia* relative to control i.e. bacteria-free medium and water treated plants (Plate 1) was probably due to the plant growth promoting traits that the strains possess, as reported by other researchers (Kifle *et al*., 2016; Tagele iet al., 2018; 2019; Yabuuch *et al*., 1995). The bacterial produced IAA was probably the main mechanism for plant growth under laboratory condition as commonly reported (Ali *et al*., 2009; Aloni *et al*., 2008; Fukaki and Tasaka, 2009; Mohite *et al.*, 2013; Zhao, 2010). Fukaki and Tasaka (20009) and Mohite *et al*. (2013) showed the increase in plant height and root elongation with increase in the amount of IAA. On the other hand, the observed increase in plant height and root elongation under screenhouse conditions could be due to the combined effects of IAA and other traits such as phosphate and zinc solubilisation and siderophore production.

However, production of indole acetic acid (IAA) and solubilisation of phosphate were the two traits which were more common among strains that demonstrated greater effects on maize growth. *Burkholderia territorii* strain KBB5, *Burkholderia sp.* QN m1 and *Ralstonia pickettii* strain r505, which were strong siderophore producers and zinc solubiliser, were observed to have relatively low effect on maize root and shoot elongation (Figure 14-15). On the other hand, the isolates *Burkholderia cepacia* strain GPY1, *Burkholderia cepacia* strain ATCC 25416 and *Burkholderia cepacia* strain GYP1, which indicated greater effect on plant growth, were observed to have relatively high phosphate solubilisation and IAA production abilities (Fig. 14-15). Thus, it is worth to conclude that indole acetic acid

production and phosphate solubilisation were the major plant growth promoting mechanisms which increased maize growth. Findings in our study match the findings reported in the study by Akinrinlola *et al*. (2018), who showed that phosphate solubilisation and IAA production were major mechanisms employed by efficient plant-growth promoting bacteria.

3.6 Conclusion

Bacterial strains were observed to increase plant growth under soil and soil-less plant growth conditions. IAA production and phosphate solubilisation were regarded as the major mechanisms even though other mechanisms could have contributed to plant growth promotion. The plant growth promotion potential exhibited by most of *Burkholderia spp* gives a clue for the possible use of these isolates as inoculant to increase plant growth and crop production. However, further investigations on effectiveness of these strains under uncontrolled conditions, including field trials, are needed before recommending the strains for commercial use.

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CHAPTER FOUR

4.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

The research presented here aimed at isolating and characterising phosphate and zinc solubilising bacteria from root surfaces of selected crops around Morogoro municipality, Tanzania, and their effects on growth of maize (*Zea mayse*). The conclusions that can be made from results of the study are that P and Zn solubilisation seem to be distributed variously in different types of microorganisms that could be exploited to improve plant growth and increase crop yield.

4.2 Recommendations

In the view of the results of the studies, the following are recommended:

i. Zinc and phosphate solubilisation was assumed to be carried out mainly through organic acids production. However, this is not always common in some strains, and furthermore it was not confirmend in this study. Therefore, further studies are needed to understand the specific mechanisms involved in solubilisation by particular strains.

ii. Microbial-plant growth promotion involves several mechanisms other than IAA and siderophore production, and zinc and phosphate solubilisation, as covered in this study. Therefore, further investigation on other mechanisms that could lead to better development of specific biofertilisers should be undertaken.

iii. Studies both in laboratory and screenhouse were conducted based on controlled environmental comditions. However, for commercial use of these bacteria as

inoculants, more studies under uncontrolled field conditions must be carried out to evaluate the survival, proliferation, adaptability in diverse environments and, consequently, the effectiveness of these strains in such environments before adoption for commercial use.